Ex ovo RNAi for Functional Gene Analysis During Neural Development

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Summary

To study gene function *in vivo* during development of the central nervous system (CNS) efficient model systems that allow for temporal and spatial control of gene expression are required. In our lab, we have used the chicken embryo in combination with *in ovo* electroporation and RNA interference (RNAi) for gene silencing during early stages of nervous system development. In particular, we used dorsal commissural neurons to study axonal pathfinding. These neurons extend their axons toward the floor plate, the ventral midline of the spinal cord. Guidance cues derived from the floor plate, the intermediate target of these axons, are important for axon growth toward and across the midline. Using *in ovo* RNAi, we could show that interference with the function of the transmembrane glycoprotein Endoglycan (PODXL2) resulted in the failure to turn or in erroneous caudal turns after midline crossing. Furthermore, the morphology of the floor plate was severely disturbed in the absence of Endoglycan. During later stages of neural development, Endoglycan is expressed by Purkinje cells in the cerebellum. For the functional characterization of Endoglycan during cerebellar development, we extended the experimental accessibility of chicken embryos to much older stages. We established the procedure of *ex ovo* electroporation and RNAi to manipulate gene expression in the developing cerebellum. The cerebellum represents a well characterized neuronal structure, and therefore, an ideal system to study CNS development, including neurogenesis, differentiation, migration, axon guidance, as well as synapse formation. To demonstrate the applicability and the efficiency of *ex ovo* RNAi, we analyzed the function of the cell adhesion molecule Axonin-1 in cerebellar development. Axonin-1 is expressed by postmitotic granule cells at the time when they extend their axons, the parallel fibers. In the absence of Axonin-1 the arrangement of granule cell axons within the molecular layer was aberrant and fibers no longer extended parallel but towards the cerebellar surface. The effect of Axonin-1 was not on parallel fiber elongation but affected specifically parallel fiber navigation. The same effects on parallel fiber development observed after *ex ovo* RNAi were reproduced in embryos treated with function-blocking anti-Axonin-1 antibodies, indicating that *ex ovo* RNAi efficiently and reproducibly silenced axonin-1 in the developing cerebellum. Thus, we used *ex ovo* RNAi to study the function of Endoglycan during cerebellar development. Endoglycan is expressed by Purkinje cells at the time when they migrate towards the pial surface to establish the Purkinje cell monolayer. Interference with Endoglycan function caused a severe migration defect of Purkinje cells. Moreover, due to the aberrant formation of the Purkinje cell monolayer, the thickness of the external germinal layer and the proliferation rate of granule cells were significantly reduced causing disturbed foliation and reduced size of the cerebellum.

Zusammenfassung

Für die Analyse von Genfunktionen während der Entwicklung des zentralen Nervensystems (ZNS) werden effiziente Systeme benötigt, welche es ermöglichen, die Genexpression zeitlich und räumlich zu kontrollieren. In unserem Labor benutzen wir *in ovo* RNAi (RNA interference), um im Hühnerembryo während früher neuraler Entwicklungsstadien Gene gezielt auszuschalten. Anhand dorsaler Kommissuralneurone studieren wir Wegweisermoleküle, welche Axone zu ihren Zielzellen dirigieren. Axone der Kommissuralneurone wachsen gegen die ventrale Mittelline des Rückenmarks, die Bodenplatte, überqueren diese und wachsen anschliessend in rostraler Richtung weiter. Wegweisermoleküle, welche direkt auf der Bodenplatte lokalisiert sind oder von dieser sezerniert werden, sind wichtig für die korrekte Wegfindung dieser Axone. Mittels *in ovo* RNAi konnten wir zeigen, dass das Transmembran-Glykoprotein Endoglycan (PODXL2) nach dem Überqueren der Bodenplatte für die anschliessende rostrale Drehung von Kommissuralaxonen wichtig ist. Der Verlust von Endoglycan-Aktivität führte zu Stillstehen am Ausgang der Bodenplatte und fehlerhaftem caudalen Wachsen von Kommissuralaxonen. Das Blockieren von Endoglycan führte ausserdem zu einer Veränderung der Morphologie der Bodenplatte. Während späterer Entwicklungsstadien wird Endoglycan von migrierenden Purkinje-Zellen des Kleinhirns (Cerebellum) exprimiert. Um die Funktion von Endoglycan im Kleinhirn zu untersuchen, haben wir mit *ex ovo* RNAi eine Methode entwickelt, welche das gezielte Hemmen einzelner Gene während fortgeschrittener Stadien der Entwicklung des Nervensystems ermöglicht. Die neuronale Struktur des Kleinhirns ist gut charakterisiert und daher ein ideales System um verschiedene Entwicklungsschritte des Nervensystems zu studieren. Um die Anwendbarkeit und Effizienz von *ex ovo* RNAi auf unsere Fragestellung zu testen, haben wir zunächst die Funktion des Zelladhäsionsmoleküls Axonin-1 im Kleinhirn untersucht. Während des Auswachsens der Axone, den so genannten Parallelfasern, exprimieren Körnerzellen Axonin-1. Der Verlust von Axonin-1-Aktivität hat zur Folge, dass die Organisation der Parallelfasern in der Molekularschicht des Kleinhirns defekt ist. Dieser Defekt beruht auf der fehleraften Navigation von Parallelfasern. Den gleichen Effekt auf die Entwicklung der Parallelfasern konnten wir mittels Injektionen von funktionsblockierenden Antikörpern erzielen. Dies deutet darauf hin, dass *ex ovo* RNAi effizient und reproduzierbar Axonin-1-Aktivität im Kleinhirn hemmen kann. Daher verwendeten wir diese Methode für die Untersuchung von Endoglycan im Kleinhirn. Dieses Gen wird von Purkinje-Zellen während ihrer radialen Migration in Richtung Oberfläche des Kleinhirns exprimiert. Das Fehlen von Endoglycan verursachte einen Migrationsdefekt der Purkinje-Zellen und eine anormale Ausbildung der Purkinje-Zellschicht. Dies wiederum führte zu einer verminderten Dicke der Körnerzellschicht bedingt durch eine Reduktion der Zellteilungsrate.

1. Introduction

The complexity of the central nervous system (CNS) reflects its comprehensive set of functions. Within a very short time it detects a tremendous amount of internal and external information, selects between relevant and irrelevant information, processes and interprets the information in order to generate and control appropriate instructions in a context-dependent manner. This multifunctional competence of the CNS requires a highly complex organization and connectivity. No other organ is made of so many different cell types, or requires such a high connectivity. In order to form a functional nervous system neurons pass through different developmental steps in a constantly changing environment until they achieve their final destination within a highly connected network consisting of more then 10^{11} neurons. Once a neuron is born, its identity, accurate position and its connectivity with proper interaction partner(s) is established by a huge arsenal of inductive signals and guidance cues. The disruption of only one or several of these developmental processes can lead to fatal consequences for brain function. Therefore, a general long-term goal of developmental neurobiology is to get a better understanding of the underlying mechanisms involved in CNS development. With new insights from basic research new drugs, therapies or strategies for medical applications can be developed. Our focus of interest is to uncover the molecular mechanisms involved in the establishment of neural circuits. What are the key players of axonal navigation required for the correct wiring of the nervous system? And how do they contribute to the accurate guidance of axons to their appropriate targets? In order to characterize the function of candidate axon guidance cues *in vivo* we use the chicken embryo as model organism because of its easy accessibility during development and the availability of tools to manipulate gene expression. The following introduction gives a summary of mechanisms implicated in axon guidance, introduces two model systems used for axon guidance studies, and discusses vertebrate model organisms used for functional gene analysis *in vivo* during embryonic development.

1.1 Mechanisms of axon guidance

During development of the central nervous system neurons extend their axons, often over long distances, in a constantly changing embryonic environment to establish functional connections with their appropriate targets. Over a century ago, the father of modern neurobiology, Ramón y Cajal reported that growing axons are tipped with a highly dynamic structure exploring its environment, the growth cone (Cajal, 1890a). In 1963, Sperry suggested that growth cones sense gradients of guidance cues and thereby direct the growing axons towards their targets (Sperry, 1963). More then 40 years later we know that steering of the growth cone is a highly complex process where external signals from the environment are transformed into an actin-based motility. During early CNS development the first developing axons grow through an axon-free environment towards specialized cells or intermediate targets that provide extracellular cues or trophic factors to ensure accurate navigation or survival of developing axons. A well characterized intermediate target of axon guidance is the ventral midline of the nervous system. This structure and the molecules involved in commissural axon guidance are discussed in chapter 1.3.1. The extracellular cues can either act as outgrowth-promoting substrates via preferred adhesion or in an instructive manner. Axon guidance requires the integration of local adhesive interactions of the substrate with the directional information from 'classical' guidance cues. However, these actions cannot strictly be separated since adhesion molecules can also provide directional information. The mechanisms by which a guidance cue contributes to the navigation of an axon can be classified into for categories: chemoattraction, contact attraction, chemorepulsion, and contact repulsion (Figure 1.1; Tessier-Lavigne and Goodman, 1996; Dickson, 2002; Chilton 2006). A short summary of the best studied families of 'classical' guidance molecules and their principle roles during CNS development is given in chapter 1.2. These four modes of action are not very strictly associated to a certain family of cues. Guidance cues are multifunctional either acting as attractant or repellent, over long or short distances, depending on the cellular and developmental context (Huber et al., 2003). A single growth cone can respond to the same cue in different ways, in particular when it is piloting intermediate targets until the final target is reached. The plasticity of guidance responses is based on the repertoire of receptors present at the cell surface. Furthermore, responses can vary due to alternative intracellular signaling, e.g. regulation of cyclic nucleotides levels and local translation in the growth cone (Song et al., 1997; Song et al, 1998; Brittis et al., 2002; Ming et al., 2002). Furthermore, the effects of guidance molecules on growth cone behavior do not happen in isolation. In fact, the cooperation of these mechanisms acting simultaneously in a temporally and spatially controlled manner provides the basis for the complexity of neural wiring. Therefore, the challenge of today's research is to understand the interactions of a relatively small number of cues together with the identification of new candidate molecules.

In addition to the 'classical' guidance molecules that have been known for quite some time, more recently morphogens which are well known for their effects on early patterning of embryonic tissue are becoming increasingly implicated in axon guidance (see 1.2.2; Bovolenta, 2005; Chilton, 2006; Stoeckli, 2006).

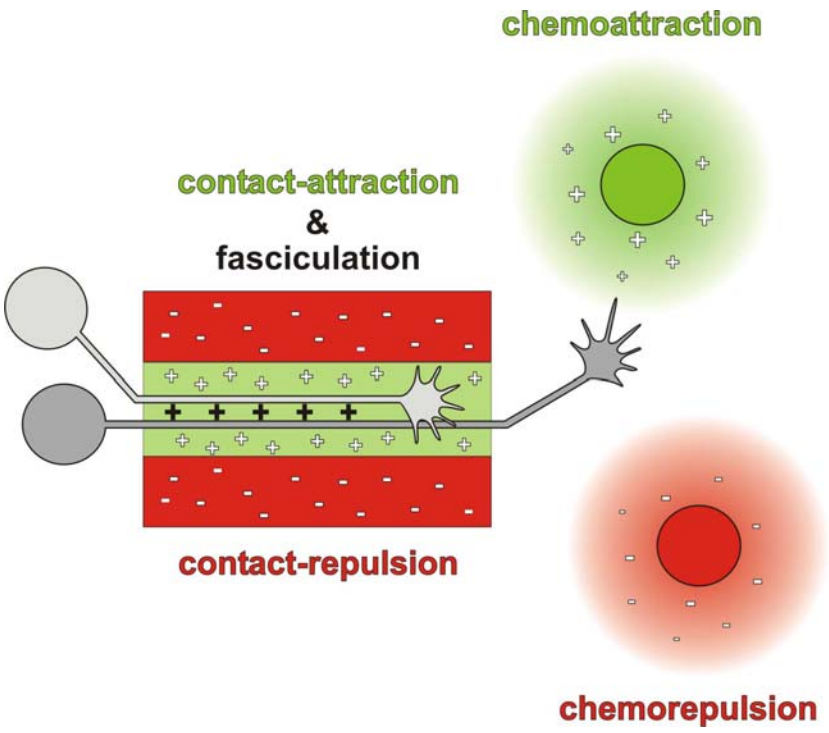

Figure 1.1 Mechanisms of guidance that contribute to the navigation of a growing axon. During nervous system development growing axons are directed by an array of guidance molecules which attract/permit (green) or repel/inhibit (red) growth cones on their journey. Both attractants and repellents can act over short (contact-mediated) or long distances (mediated by chemotaxis). Additionally, fasciculation between axonal tracts (bold+) and defasciculation (not shown) can support accurate axon pathfinding. These mechanisms cannot strictly be allocated to a certain family of guidance molecules, because guidance cues are not exclusively attractive or repulsive but rather bifunctional depending on the developmental context. Furthermore, some families of guidance molecules have membrane-bound/non-diffusible and secreted/diffusible members that are acting over short or long distances, respectively.

During later stages of neuronal development when initial tracts have been formed axon guidance can be facilitated by those pioneer tracts. The so called pioneer axons that developed during early stages of development can serve as scaffolds that will be followed by later developing axons at a time when the embryonic environment has become more complex. Therefore, axon guidance can be supported by selective fasciculation between developing axons and preexisting pioneer axons. This process requires specific recognition between two appropriate populations of axons (Raper et al., 1983; van Vactor, 1998).

1.2 Molecules implicated in axon guidance and cell migration

1.2.1 'Classical' axon guidance molecules

Ephrins and Eph receptors

The topographic mapping in the chicken visual system served Sperry as a model to investigate the underlying nature of the highly accurate organization of retinal ganglion cell projections into the tectum. Axons from the temporal retina terminate in the anterior tectum in a stereotyped manner whereas nasal retinal axons project into the posterior tectum. He postulated in his chemoaffinity hypothesis that retinal axons are guided to their appropriate tectal areas by a system of molecular gradients (Sperry, 1963). The search for these graded cues led to the identification of the Ephrins (Figure 1.2). Ephrins are membrane-bound proteins either attached to the cell surface via glycosylphosphatidylinositol anchor (Ephrin-As) or via transmembrane domains (Ephrin-Bs; Cheng et al., 1995, Drescher et al., 1995). EphrinAs and EphrinBs interact with their receptors EphAs and EphBs, respectively (Cutforth and Harrison, 2002; Pasquale, 2005). While EphrinA/EphA are expressed in opposite antero-posterior gradients in the tectum and retina mediating repulsion of retinal axons (Monschau et al., 1997), EphrinB/EphB are expressed along the dorso-ventral axis and attract retinal axons (Hindges et al., 2002; Mann et al., 2002). The combination of these two opposite gradients provides positional information for retinal axons to innervate the tectum in a topographic manner.

In contrast to the vertebrate visual system where Ephrins act as ligands and Ephs as receptors, for axons of the anterior commissure EphrinB functions as receptor leading to repulsive effect on these axons (Henkemeyer et al., 1996). Because of the possibility for 'reverse' or 'bi-directional' signaling it is no longer useful to call Ephs receptors for Ephrin ligands. Rather these molecules should be regarded as binding partners in axon guidance.

Semaphorins and Plexin/Neuropilin receptors

Originally, Semaphorins have been identified as molecules with growth-cone collapsing activities (Luo et al., 1993). Semaphorins comprise a large family consisting of 8 classes of membrane-bound and secreted guidance cues with a characteristic ~420-amino acid-long Sema domain. They act mainly as repellents (Figure 1.2; Luo et al., 1995; Messersmith et al., 1995; Kobayashi et al., 1997; Koppel et al., 1997; Chedotal et al., 1998; Zou et al., 2000). Classes 1 and 2 are found in invertebrates, classes 3 to 7 are found in vertebrates, and class V Semaphorins are encoded by viruses (Raper, 2000). They signal through multimeric receptor complexes formed by Plexins and Neuropilins, although the composition of theses complexes is not fully uncovered. Plexins are a large family of four classes of transmembrane proteins with signaling function whereas Neuropilins seem to contribute to binding

specificity (Nakamura et al., 1998; Takahashi et al., 1999; Tamagnone et al., 1999; Tamagnone and Comoglio, 2000). Besides Plexins and Neuropilins as binding partners for Semaphorins additional proteins were shown to be part of the receptor complex, e.g. the cell adhesion molecule L1 or the receptor tyrosine kinase Off-track (Otk) play an important role in Semaphorins' repulsive function on migrating axons (Castellani et al., 2000; Winberg et al., 2001).

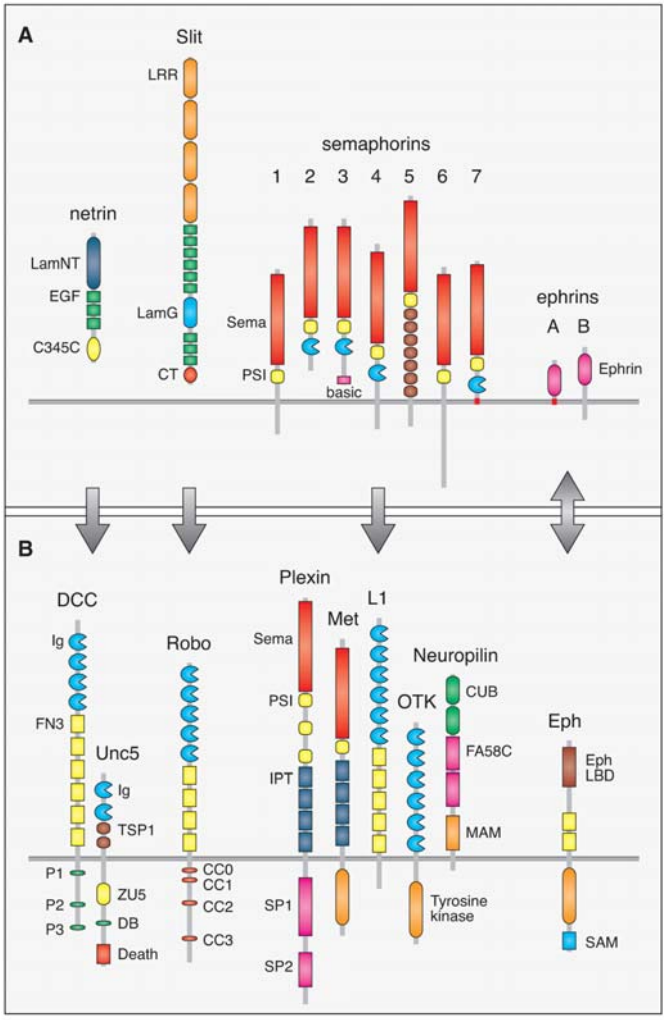

Figure 1.2 Conserved families of 'classical' guidance molecules (A) and their receptors (B). 'Classical' guidance molecules comprise 5 families of molecules including the Netrins, Slits, Semaphorins, Ephrins, and the cell adhesion molecules of the immunoglobulin superfamily (IgSF CAMs; not shown). The secreted Netrin acts via its receptors DCC, Unc5, or the combination of DCC and Unc5. The effect of the secreted protein Slit is mediated by Robo receptors. Semaphorins comprise a large family of membrane-bound and secreted guidance cues signaling through multimeric receptor complexes formed by Plexins, Neuropilins, L1, and OTK. Ephrins act through Ephs or vice versa allowing for 'bi-directional signaling. Domain names are from SMART (http://smart.embl-heidelber.de). P1 to P3, DB (DCC-binding), CC0 to CC3, and SP1 and SP2 indicate conserved regions in the cytoplasmic domains of DCC, UNC-5, Robo, and Plexin receptors, respectively (from Dickson, 2002).

Netrins, DCC, and Unc-5 receptors

The search for a chemoattractant for vertebrate commissural axons led to the identification of the Netrins (see also chapter 1.3.1 and Figure 1.2; Kennedy et al., 1994; Serafini et al., 1994). Netrins are phylogenetically conserved secreted proteins with sequences homology to the proteins of the laminin family (Hedgecock et al., 1990; Serafini et al., 1994). Independent functional studies in *C. elegans*, *Drosophila*, and vertebrates demonstrated that the Netrins/Unc-6 can exert both an attractive or repulsive effect depending on the receptors involved and the neuronal populations concerned (Hedgecock et al., 1990; Ishii et al., 1992; Harris et al., 1996; Mitchell et al. 1996; Barallobre et al., 2000). Netrins attract commissural axons (Serafini et al., 1996), thalamocortical axons (Braisted et al., 2000), and dopaminergic axons (Shewan et al., 2002), but repel trochlear motor axons (Colomarino and Tessier-Lavigne, 1995). For retinal axons it has been shown that axons may change their responsiveness to Netrin from attraction to repulsion as they advance along the pathway (Shewan et al., 2002). Whereas DCC (Deleted in Colorectal Cancer)/Unc-40 functions as Netrin-1 receptor to trigger an attractive effect, Unc-5 alone or in combination with DCC is required for the repulsive effect of Netrin (Keino-Masu et al., 1996; Hong et al., 1999; Keleman and Dickson, 2001; Mehlen and Mazelin, 2003). In addition to DCC and Unc-5, Neogenin binds to Netrin (Wang at al., 1999). However, in contrast to Netrin/DCC signaling and RGM (repulsive guidance molecules)/Neogenin signaling (Rajagopalan et al., 2004), a function for Netrin/Neogenin signaling in axon guidance remained unknown until recently. Wilson and Key provided first experimental data that Netrin interacts with Neogenin during axon guidance in the *Xenopus* forebrain (Wilson and Key, 2006). Although the functions of Netrin as long- or short-range cue have been known for quite some time, evidence for a Netrin gradient or an explanation of how this molecule diffuses away from its source within the embryonic tissue has been reported only recently (Kennedy et al., 2006).

Slit and Robos

Based on genetic screens for midline guidance defects in *Drosophila* the Roundabout (Robo) receptor and its ligand Slit have been identified (Figure 1.2; Seeger et al., 1993; Kidd et al., 1999). The repulsive cue Slit is expressed at the ventral midline and prevents ipsilateral axons from crossing the midline and commissural axons from recrossing via the receptor Robo (see also chapter 1.3.1; Battye at al., 1999; Kidd et al., 1999). In *Drosophila*, Slit/Robo signaling on commissural axons is regulated by Commissureless (Comm), although the exact mechanism is not completely understood (Georgiou and Tear, 2002; Keleman et al., 2002; Keleman et al., 2005). The repulsive action of Slit at the ventral midline is conserved in vertebrates (Brose et al., 1999; Zou et al., 2000). In vertebrates three Slits and Robos have been identified (Brose et al., 1999; Long et al., 2004). However, a vertebrate homologue for Comm is missing. Therefore, the search for a plausible mechanism regulating Slit-Robo signaling at the ventral midline is still under extensive investigation. A potential candidate that takes over Comm function in vertebrates is Robo3/Rig1. It is highly expressed by commissural axons before midline

crossing and in mice lacking Rig1 axons failed to cross the midline, indicating that Rig1 may mask the action of Slit and therefore prevent premature repulsion by Slit (Sabatier et al., 2004). Alternatively, the insertion of Robo receptors at the cell surface might be a regulatory mechanism for Slit repulsion. Based on a screen for guidance cues involved in midline crossing we have identified Rab GTPase guanine nucleotide dissociation inhibitor (RabGDI), an important component of the vesicle trafficking machinery. Perturbation of RabGDI in commissural axons copied Slit and Robo phenotypes at the floorplate. Furthermore, *in vitro* data provide evidence that Robo1 translocation to the cell surface depends on RabGDI (Philipp et al., submitted). In addition to Slit repulsion after midline crossing, it has been shown that commissural axons become responsive to Semaphorins which increases the repulsion away from the midline (Zou et al., 2000). Furthermore, Robo can silence the attractive effect of Netrin through direct interaction of Robo and DCC (Stein and Tessier-Lavigne, 2001). Although the Slit-Robo System has been mainly analyzed during commissural axon guidance at the ventral midline, it is also involved in guiding retinal axons at the optic chiasm and stimulates sensory axon branching and elongation (Wang et al., 1999; Plump et al., 2002).

Cell adhesion molecules (CAMs)

Members of the immunoglobulin (Ig) and Cadherin superfamily are well known for their function during axon pathfinding and fasciculation (Tessier-Lavigne and Goodman, 1996; Stoeckli et al., 1997; Stoeckli and Landmesser, 1998; Song and Poo, 2001). These cell adhesion molecules can either mediate homophilic or heterophilic interactions by functioning as receptors or ligands. Depending on the combination of CAM interactions different responses of the same neuronal population are evoked (Stoeckli and Landmesser 1995). For instance, perturbation of Axonin-1/NgCAM interaction results in defasciculation of dorso-ventrally growing commissural axons without effecting their growth direction towards the floor plate. In contrast, after blocking Axonin-1/NrCAM interaction commissural axons failed to cross the ventral midline und turned rostrally on the ipsilateral border of the floor plate (Stoeckli and Landmesser 1995). In addition to a direct guidance function CAMs can also modify the effect of other guidance cues (Castellani et al., 2000; Bechera et al., 2007; Maness and Schachner, 2007)

In chapter 2, I discuss the function of Axonin-1/TAG-1 during development of cerebellar granule cells. Although Axonin-1/TAG-1 was used as a marker for postmitotic granule cells, its function in the cerebellum remained unknown for a long time (Kuhar et al., 1993; Hatten et al., 1997). It has been shown that Axonin-1/TAG-1 is required for the guidance of sensory axons and commissural axons *in vivo* (Stoeckli and Landmesser 1995; Perrin et al., 2001). Using *ex ovo* RNAi and *in vitro* approaches we demonstrate that Axonin-1/TAG-1 is required for the navigation of granule cell axons rather then for their elongation.

1.2.2 Morphogens

A common theme of axon pathfinding and tissue patterning is that instructive signals are provided in a graded manner. Morphogens are secreted signaling molecules localized as gradients within the tissue that are well known for their function in controlling cell fate and tissue patterning (Jessell, 2000). Over the last years an important role in axon guidance was shown for morphogens, such as the bone morphogenetic proteins (BMPs), the Wnt family, and Sonic hedgehog (Shh). Besides patterning the dorsal spinal cord, BMPs repel commissural axons away from the dorsal midline (Augsburger et al., 1999) in cooperation with the chemoattractive effect of Netrin (Kennedy et al., 1994; Serafini et al., 1996). In *Drosophila* Wnt5 and its receptor Derailed (Drl) are involved in axon guidance at the ventral midline (Yoshikawa et al., 2003). In mice, Wnt4 acts a chemoattractant for commissural axons after midline crossing directing them rostrally via the Frizzled receptor (Lyuksyutova et al., 2003). The role of Shh during postcommissural axon guidance was one of our main interests during the last years. During early embryonic development Shh acts as a morphogen involved in inductive and patterning processes (MartÍ and Bovolenta, 2002; Jacob and Briscoe, 2003). Later on, Shh secreted from the floor plate exerts an attractive effect on commissural axons in parallel to Netrin-1 (Charron et al., 2003). Finally, as shown in our lab, after crossing the floor plate Shh directs commissural axons rostrally along the longitudinal axis in a repulsive manner (Bourikas et al., 2005). Interestingly, the last function of Shh is mediated by HIP (Hedgehog interacting protein) and not by Patched and Smoothened (Bourikas et al., 2005). The example of Shh demonstrates that one signaling molecule does not act in isolation and can switch between different functions within a short time window depending on the developmental context.

1.2.3 Extracellular matrix (ECM) molecules

In contrast to the ‘classical’ axon guidance molecules and morphogens that direct axonal outgrowth, many ECM molecules, including Laminin, Fibronectin, Tenascin, as well as Vitronectin are well known for their support or inhibition of neurite outgrowth *in vitro* (Bixby and Harris 1991). However, a clear separation between outgrowth promotion and axon navigation cannot be made. Indeed, a combination of local adhesive interactions with the ECM or neighboring cells and directional instructions provided by cues is required for axon guidance. Receptors for ECM molecules are mainly Integrins, members of the Ig superfamily and proteoglycans (see also 1.2.4). Although little is known about actual guidance functions of ECM molecules, an ‘indirect’ effect on axon guidance has been shown. Höpker and colleagues demonstrated that in *Xenopus* Laminin converts Netrin-mediated attraction into repulsion for retinal neurons (Höpker et al., 1999). Additionally, for Laminin and Fibronectin it has been shown that they can modulate the action of secreted Frizzled related protein 1 (SFRP1), a proposed Wnt signaling inhibitor (Rodriguez et al., 2005). Although the underlying mechanisms are still unknown, these findings indicate that ECM molecules not only promote axon outgrowth, but also modify the behavior of growth cones in response to guidance cues.

1.2.4 Glycoproteins

Heparan sulfate proteoglycans (HSPGs) are complex macromolecules consisting of polysaccharide side chains attached to a core protein. Recently, HSPGs have been found to be involved in cell migration and axon pathfinding (Holt and Dickson, 2005; Rhiner and Hengartner, 2006). The cell surface HSPGs Syndecan (transmembrane) and Glypican (GPI-anchored), which are present on neurons, can interact with their heparan sulfate chains with a variety of extracellular guidance cues and matrix proteins, and therefore can control the effect of ligand-receptor interactions. For instance, enzymatic removal of heparan sulfate chains that bind Slit causes a loss of Slit induced repulsion *in vitro* (Hu, 2001; Ronca et al., 2001). Loss of Syndecan function in *Drosophila* results in aberrant midline crossing of longitudinal axons, similar to Slit or Robo phenotypes (Johnson et al., 2004). Additional interactions with well known guidance cues, including Semaphorins and Ephrins have been reported (Ethell et al., 2001; Kantor et al., 2004). Furthermore, mice and zebrafish lacking the glycosyltransferase Ext, a key enzyme in heparan sulfate biosynthesis, showed several severe CNS defects due to abnormal patterning and axon guidance (Inatani et al., 2003; Lee et al., 2004). There are first hints that specific functions of HSPGs depend on their heparan sulfate modifications (Bülow and Hobert, 2004). In addition to the temporal and spatial control of gene expression, the enormous diversity of heparan sulfate modifications and their potential effects on guidance cues may enlarge the possibilities for instructive information underlying the formation of wiring patterns.

In chapter 3, I focus on the identification and functional characterization of the glycoprotein Endoglycan (PODXL2), a member of the CD34 family of sialomucins, during development of the chicken spinal cord and cerebellum. So far, the function of Endoglycan during development of the central nervous system was unknown. We show that Endoglycan is predominantly expressed by floor-plate cells in the developing spinal cord. Interference with Endoglycan function using *in ovo* RNAi caused aberrant floor-plate morphology and pathfinding errors of commissural axons. Additionally, Endoglycan is expressed by migrating Purkinje cells in the cerebellum later in development where it appears to play a crucial role in Purkinje cell migration. In the absence of Endoglycan Purkinje cells failed to migrate radially towards the pial surface. Due to the aberrant migration of Purkinje cells the formation of the Purkinje cell monolayer was severely disturbed causing in turn a significant reduction of the size and foliation of the cerebellum.

1.3 Model systems for axon guidance studies

1.3.1 Commissural axon guidance at the ventral midline

To reveal basic mechanisms and molecules involved in axon guidance a model is required that provides easy access and contains relatively simple and conserved structures. To minimize erroneous pathfinding over long distances axons encounter several intermediate targets along their trajectory which provide directional information. One of the best characterized intermediate target and important system that contributed much to our understanding of the molecular mechanisms of axon guidance is the ventral midline of the nervous system. In the vertebrate spinal cord and in the insect ventral nerve cord the midline serves as an intermediate target for commissural axons on their pathway through the developing nervous system. Commissural neurons belong to a population of interneurons of the neural tube that project their axons to the contralateral side to ensure information transfer between the two halves of the body in bilaterally- symmetric organisms. The cell bodies of the

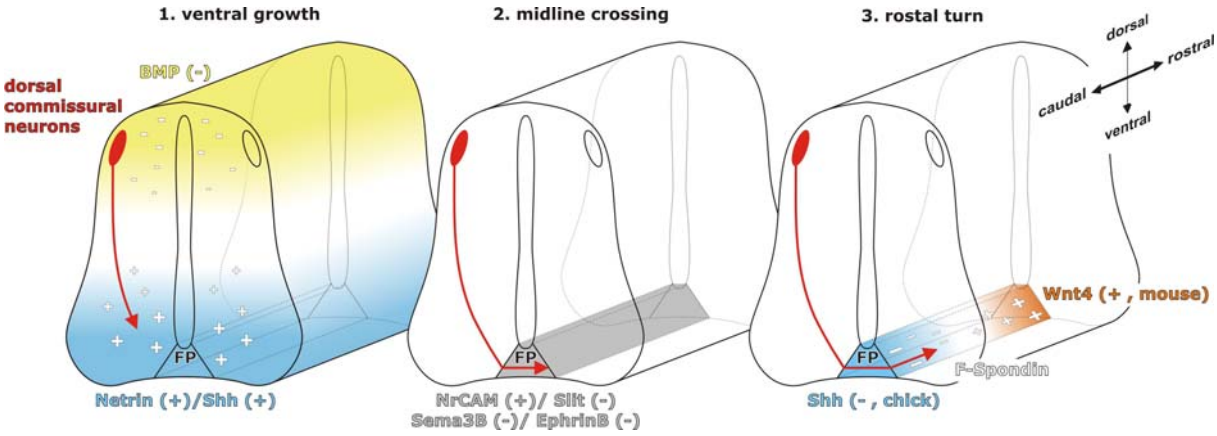

Figure 1.3 Guidance molecules involved in commissural axon guidance. Dorsal commissural neurons (red) located in the dorso-lateral part of the developing spinal cord extend their axons towards the ventral midline, called floor plate (left). After crossing the floor plate (middle) commissural axons turn rostrally along the longitudinal body axis (right). Commissural axon growth towards the floor plate is directed by a high-dorsal to low-ventral gradient of the repellent bone morphogenetic protein 7 (BMP7) and high-ventral to low-dorsal gradients of the attractants Sonic Hedgehog (Shh) and Netrin (left). Guidance of commissural axons across the floor plate is mediated by an interaction between the two cell adhesion molecules Axonin-1 on commissural axons and NrCAM on floor-plate cells. In addition, the repulsive effects of Slit, Sema3B, and EphrinB ensure that commissural axons cross the midline once and leave the floor plate on the contralateral side (middle). Finally, a high-rostral to low-caudal gradient of the attractant Wnt4 and a low-rostral to high-caudal gradient of the repellent Shh direct commissural axons rostrally after midline crossing in mice and chicken, respectively (right). Whether gradients of Wnt4 and Shh are expressed in chicken and mice remains to be elucidated. F-Spondin secreted by the floor plate controls the 90° turning angle of commissural axons at the contralateral floor-plate border. FP: floor plate, (-): repulsive effect on commissural axons, (+): attractive or promoting effect on commissural axons.

dorsal commissural neurons are located in the dorso-lateral area of the neural tube (Kadison and Kaprielian, 2004). During early development of the spinal cord, commissural neurons extend their axons towards the ventral midline consisting of columnar ependymal cells, called the floor plate. This dorso-ventral growth of commissural axons is initiated by a cooperation of BMP, Netrin, and Shh signaling (Figure 1.3; see also 1.2.1; Serafini et al., 1996; Augsburger et al., 1999; Charron et al., 2003). Subsequently, commissural axons cross the ventral midline guided via positive and negative signals derived from Axonin-1/NrCAM interactions, Sema3B, EphrinB, and Slit (see also 1.2.1; Stoeckli and Landmesser, 1998; Zou et al., 2000; Kaprielien et al., 2001; Long et al., 2004). Finally, after successful midline crossing commissural axons make a sharp turn of 90 degrees and grow rostrally along the longitudinal body axis. Although much less is known about the navigation along the anterior-posterior axis compared to the dorso-ventral axis, the floor plate is still important as no axons turn rostrally in the absence of this structure (Bovolenta and Dodd, 1991). It has been shown that F-Spondin, a protein secreted by the floor plate, is responsible for the restriction of the turning angle of commissural axons at the contralateral floor-plate border (Burstyn-Cohen et al., 1999). Recently, it has been shown that morphogens play a crucial role in postcommissural axon guidance (Stoeckli, 2006). In mouse, Wnt4 is expressed in a high-rostral to low-caudal gradient and thereby attracts postcommissural axons (Lyuksyutova et al., 2003). In the chick, Shh is expressed in a low-rostral to high-caudal gradient and thereby repels postcommissural axons (Bourikas et al., 2005). A possible interaction between Wnt and Shh signaling during postcommissural axon pathfinding remains to be elucidated and is currently under investigation in our lab.

Taken together, the commissural axons grow towards and cross the ventral midline before they turn towards the brain in a stereotypic manner. The simple axonal trajectories and the uniform behavior make them an ideal system to study axon guidance. Furthermore, spinal cord commissural axons are easily accessible allowing for their analysis *in vitro* as well as *in vivo*.

1.3.2 The cerebellum as a system to study brain development

Although the cerebellum is also known as the 'little brain', because it represents only 10% of the total brain volume, the mature cerebellum contains more than half of the brain's neurons (Wang and Zoghbi, 2001). It is not exaggerated to say that the cerebellum is a multitask unit of the brain. Besides its importance in the coordination of movements (Apps and Garwicz, 2005), it has been linked to certain cognitive processes (Chizhikov and Millen, 2003; Schmahmann, 2004,) as well as in experience-dependent adaptive and learning processes (Thach, 1998).

The cellular and laminar organization of the cerebellum as well as the cerebellar circuit is uniform and well described (Figure 1.4; Wang and Zoghbi, 2001; Apps and Garwicz, 2005). The cerebellar cortex consists of eight different neuronal cell types: the Purkinje cells, granule cells, Golgi cell, Lugaro cells,

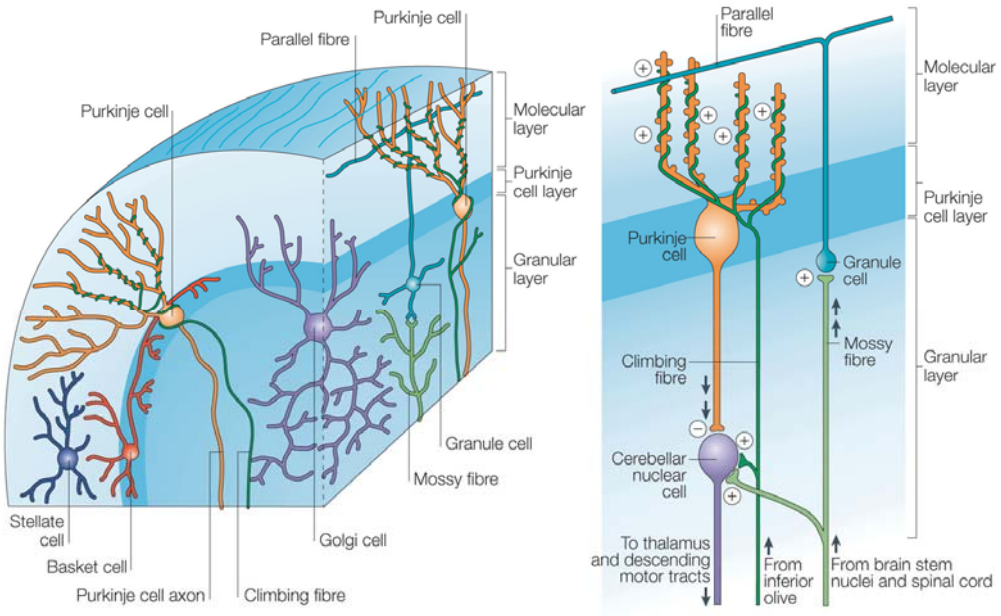

Figure 1.4 Basic organization of the cerebellar circuitry. The mature cerebellar cortex is organized in three distinct layers: the molecular layer, the Purkinje cell layer, and the inner granular layer (IGL). The outer most cerebellar layer, the molecular layer, consists of the parallel fibers, which form excitatory synaptic contacts with Purkinje cells and interneurons, namely stellate cells and basket cells. Typically, parallel fibers extend for several millimeters along the length of individual cerebellar folia. The Purkinje cells, organized as a monolayer, have the only axons which project out of the cerebellum, and therefore, provide the only cerebellar output. The IGL harbors the most abundant neuronal cell type, the granule cells, as well as Golgi cells. The cerebellum receives external information via two main afferent systems: climbing fibers which make direct excitatory contacts with Purkinje cells and mossy fibers which make excitatory synaptic contacts mainly with granule cells, but also with Golgi cells. With the exception of granule cells, all cerebellar cortical neurons, including the Purkinje cells, make inhibitory synaptic connections with their target neurons (from Apps and Garwicz, 2005).

basket cells, stellate cells, candelabrum, and unipolar brush cells (Sotelo, 2004). By far the largest neuronal population of the whole CNS are the granule cells. They are located in the innermost cerebellar layer, called inner granular layer, from where they project their axons to the most superficial molecular layer. In the molecular layer the bifurcated granule cell axons, called the parallel fibers, form synaptic contacts with the dendritic trees of the Purkinje cells (Voogd and Glickstein, 1998). At the interface of the inner granular layer and the molecular layer the Purkinje cell bodies are aligned in a single row. The Purkinje cells represent the only cerebellar output with projections to the deep cerebellar nuclei and the brain stem (De Camilli et al., 1984). Basket cells and stellate cells located in the molecular layer together with Golgi and Lugaro cells located in the inner granular layer belong to classical inhibitory interneurons of the cerebellum (Zhang and Goldman, 1996; Weisheit et al., 2006). The inhibitory candelabrum cells located in the Purkinje cell layer and the excitatory unipolar brush cells have been described as additional cerebellar interneurons (Laine and Axelrad, 1994; Mugnaini and Floris, 1994). The cerebellum receives input from two afferent systems: The climbing fibers and the mossy fibers. Originating from the inferior olive one climbing fiber directly contacts one Purkinje cell in the molecular layer (Eccles et al., 1966; Sotelo et al., 1975). In contrast to this, mossy fibers arising from various nuclei in the spinal cord, brain stem and deep cerebellar nuclei form contacts with hundreds of granule cells and therefore can affect thousands of Purkinje cells (Tolbert et al., 1978; Matsushita et al., 1979; Gould, 1980).

The cerebellum has served as a system to study mechanisms involved in neural development and has been studied in a variety of model organisms (Goldowitz and Hamre, 1998; Sotelo, 2004). During early development the neural tube comprises four different regions: The prosencephalon (subdivided in telencephalon and diencephalon), mesencephalon, rhombencephalon (subdivided in metencephalon and myelencephalon), and spinal cord. Classical chick-quail transplantation experiments demonstrated that the mesencephalon and metencephalon contribute to the developing cerebellum (Hallonet and Alvarado-Mallart, 1997). For these transplantation studies the vesicle boundary between the mesencephalon and metencephalon has been used as grafting landmarks. However, molecular markers such as Otx2 which defines the caudal limit of the midbrain and Hoxa2 which defines the caudal limit of the cerebellum do not map to the vesicular boundaries at the embryonic stages at which grafting is possible (Millet et al., 1996; Wingate and Hatten, 1999). Using molecular landmarks rather than vesicle boundaries for quail-chick fate-mapping, Wingate and Hatten showed that the most anterior part of the metencephalon, rhombomere1, exclusively gives rise to the developing cerebellum (Wingate and Hatten, 1999; Wingate et., 2001). A number of important secreted and regulatory genes (Fgf8, Wnt1, En1, En2, Pax2, Pax5, Pax6, Pax8, Otx1, Otx2, Gbx2, Wnt5a, and Nkx2.2) are expressed in the region of the isthmus, the junction between mes- and metencephalon (also called midbrain/hindbrain boundary (MHB), Goldowitz and Hamre 1998; Wang and Zoghbi, 2001). Mutations of several of these genes resulted in large deletions or complete loss of the cerebellum indicating that the isthmic constriction exerts an inductive action on neighboring neuroepithelial cells. Therefore, this region has been named ‘isthmic organizer’. Searching for a candidate morphogen released by the isthmic organizer with loss- and gain-of-function experiments resulted in the identification of Wnt1 and Fgf8. For both candidates loss-of-function resulted in a

deletion of most of the mes/met region (McMahon and Bradley, 1990; Meyers et al., 1998). However, only for Fgf8 a morphogenetic role in this region has been demonstrated, while ectopic expression of Wnt1 failed to provide experimental evidence for Wnt1 as the morphogen of the isthmic organizer (Crossley et al., 1996; Hidalgo-Sanchez et al., 1999; Martinez et al., 1999; Adams et al., 2000; Sato et al., 2001).

In contrast to other CNS regions, cerebellar neurons and interneurons originate from two distinct proliferative zones (Figure 1.5). The ventricular neuroepithelium gives rise to Purkinje cells, deep nuclear neurons, Golgi neurons, and interneurons of the molecular layer (stellate and basket cells) whereas granule cell precursors originating from the rhombic lip proliferate in the external germinal layer (EGL; Zhang and Goldman, 1996; Hatten and Heintz, 1995; Hatten, 1999). During cerebellar development Purkinje cells exit the ventricular zone after their last mitosis and migrate towards the pial surface to form the Purkinje cell monolayer. Although a mouse mutant with defective Purkinje cell migration (*reeler*) has been reported a long time ago, the actual function of the affected gene, *reelin*, during this process is still poorly understood (Mariani et al. 1977; Miyata et al., 1997; Pearlman et al., 1998; Magdaleno et al., 2002).

Granule cell precursors from the rhombic lip, the interface between the ventral roof of the forth ventricle and the neural tube, migrate tangentially onto the surface of the developing cerebellum to form a secondary germinal region, the external germinal layer (EGL; Ryder and Cepko, 1994; Alder et al., 1996; Wingate and Hatten, 1999; Wingate, 2001). *In vivo* data suggest that the tangential migration of granule cell precursors is in part mediated by Netrin/Unc5 signaling (Wingate, 2001). Extensive *in vivo* studies revealed that granule cell proliferation in the outer EGL is controlled by several secreted proteins such as BMP, SDF-1, and SHH (Alder et al., 1999; Dahmane and Ruiz I Altaba, 1999; Wechsler-Reya and Scott, 1999; Klein et al., 2001). Subsequently, granule cells differentiate and start to extend their axons, the parallel fibers, in the inner EGL. Recently, it has been shown that the transmembrane protein Plexin-B2 is involved in keeping the balance between granule cell proliferation in the outer EGL and differentiation in the inner EGL (Friedel et al., 2007). In chapter 2 we show that the navigation of granule cell axons, the parallel fibers, depends on the cell adhesion molecule Axonin-1/TAG-1. After initial parallel fiber extension within the developing molecular layer granule cells develop a third process, perpendicular to the pial surface, and guided by Bergmann glia cells start to migrate radially through the Purkinje cell layer (PCL) to form the inner granular layer (IGL; Yacubova and Komuro, 2003). The switch from tangential migration within the inner EGL to the glial-guided radial migration towards the IGL is initiated by Semaphorin6A in a non-cell autonomous manner (Kerjan et al., 2005). Finally, parallel fibers of granule cells form synaptic contacts with dendrites of Purkinje cells within the molecular layer (ML). Up to now, synaptogenesis between granule and Purkinje cells remains a black box. However, it has been shown that Wnt7A signaling is involved in synapse formation between granule cells and mossy fibers (Hall et al., 2000). Whether Wnt signaling plays a similar role in the molecular layer has to be elucidated. Later on, the EGL

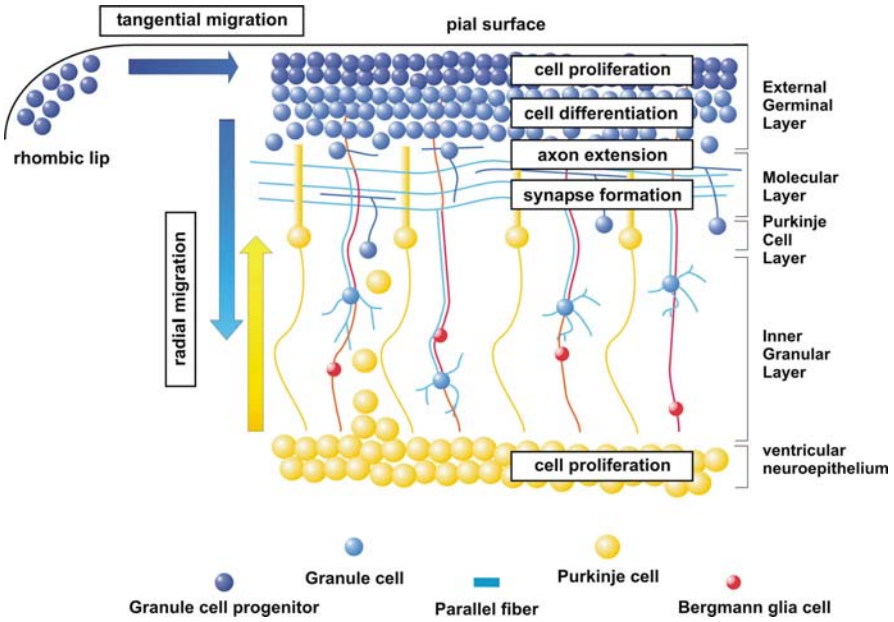

Figure 1.5 Development of the cerebellum. Cerebellar neurons and interneurons emerge from two proliferative zones: The ventricular neuroepithelium and a secondary germinal zone located at the pial surface, called the external germinal layer (EGL). The ventricular neuroepithelium gives rise to Purkinje cells (yellow), Bergmann glia cells (red) and cerebellar interneurons (not shown). After proliferation in the ventricular zone, Purkinje cells exit from the cell cycle and migrate radially towards the pial surface to establish the Purkinje cell monolayer which is established at around stage HH38 in the chicken embryo. Once Purkinje cells are settled down as a monolayer they start to build synaptic trees into the developing molecular layer. Granule precursor cells (blue) originate from the rhombic lip, the roof of the forth ventricle, and migrate tangentially onto the outer surface of the developing cerebellum to form the EGL. After massive proliferation in the outer EGL, granule cells differentiate in the inner EGL and start to extend their axons, the parallel fibres, in the developing molecular layer where they form synaptic contacts with the dendritic trees of Purkinje cells. After initial axon extension, guided by Bergmann glia cells granule cells migrate radially through the Purkinje cell monolayer to settle in the inner granule cell layer. Finally, the EGL ceases to exist and the mature cerebellum is organized in molecular layer, Purkinje cell layer and IGL.

disappears and the mature cerebellar cortex is finally organized in three distinct layers: The molecular layer, the Purkinje cell layer and the inner granular layer.

Taken together, the cerebellum represents a neuronal structure with intermediate anatomical complexity compared to the spinal cord and the cerebrum. Its uniformity and simple network consisting of a small number of different neuronal cell types makes the cerebellum an ideal system to study the molecular regulation of basic developmental processes including cell proliferation, neuronal differentiation, cell migration, axon extension, and synapse formation. Therefore, it does not surprise that the study of the cerebellum allowed Cajal more than one hundred years ago to formulate the ‘Neuron Theory’, a milestone of modern neurobiology (Cajal, 1890b). Although the organization and the development of the cerebellum are well characterized at the morphological level, the underlying molecular machinery is still poorly understood. A better understanding of these processes will not only

provide valuable insight into CNS development, but also general insights into CNS diseases ranging from tumor formation to cerebellar malformations and neurodegeneration (see also chapter 4; Berglund et al., 1999; Wechsler-Reya, 2003; Serra et al., 2006).

Compared to rodents where cerebellar development continues postnatally, the chicken cerebellum is fully developed und functional at the time of hatching. In the chicken, after 21 days of embryonic development, the formation of the cerebellum is completed. The developing chicken cerebellum is easily accessible for experimental manipulations when embryos are cultured in a shell-less way, *ex ovo*. Therefore, one goal of my PhD thesis was to establish an efficient tool for functional gene analysis *in vivo* using the chicken cerebellum as a model system. Based on our technique for gene silencing during early stages of the nervous system development in the chicken embryo - *in ovo* RNAi - we have established *ex ovo* electroporation and *ex ovo* RNAi to manipulate gene expression during later stages of brain development, allowing for specific gene silencing in the developing cerebellum (Chapter 2). Using *ex ovo* RNAi, we could demonstrate that the immunoglobulin superfamily cell adhesion molecule Axonin-1/TAG-1 is required for proper parallel fiber extension. Furthermore, we have used this technique for the functional characterization of the transmembrane glycoprotein Endoglycan during cerebellar development (Chapter 3).

1.4 Functional gene analysis *in vivo*

1.4.1 Introduction

Due to the sequencing of several vertebrate genomes the functional analysis of genes in the context of a living organism rather than the identification of candidate genes has become the challenging step of today's research. High-throughput methods have been developed for the analysis of gene expression but unfortunately they do not provide any insight into the function of these genes. Therefore, efficient model systems for studying gene function *in vivo* by gain- and loss-of-function approaches are required. In particular, for neurodevelopmental studies the temporal and spatial control of gene expression becomes extremely important for the functional characterization of a candidate gene. As mentioned above, during development of the nervous system neurons pass through a sequence of complex processes to form a functional nervous system. The coordination of these processes requires a tightly regulated program of precise spatio-temporal gene expression. Many genes can exert different functions at distinct time points of development.

Developmental neurobiology is a very diverse field as are the experimental approaches used. Depending on the researcher's focus and interest the ideal model system has to be selected. The vertebrate model organism of choice has to fulfil several criteria including availability of techniques for gene manipulation as well as easy accessibility during development. In addition, costs for maintenance, expenditure of time, and ease of handling of a model organism should be considered.

The following review summarizes available techniques of gene manipulation in vertebrate animal models and discusses their advantages as well as drawbacks concerning temporal and spatial control of gene expression during neural development. The main focus is on the chicken embryo as model organism and *in ovo* RNAi as an efficient technique for temporal and spatial control of gene silencing *in vivo*.

1.4.2 *In ovo* RNAi opens new possibilities for temporal and spatial control of gene silencing during development of the vertebrate nervous system

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key words: RNAi, chicken embryo, in ovo RNAi, in utero RNAi, nervous system, embryonic development

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REVIEW ARTICLE

In ovo RNAi opens new possibilities for temporal and spatial control of gene silencing during development of the vertebrate nervous system

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ABSTRACT

Loss-of-function approaches are important tools for functional gene analysis. Due to the availability of sophisticated methods to manipulate gene expression in embryonic stem cells that can be used to generate mutant mice, the mouse is by far the most important vertebrate model organism for basic and applied biomedical research. Unfortunately, the available methods do not allow for precise temporal and spatial control of gene silencing during embryonic development limiting the usefulness of the mouse for developmental studies. Due to their easy accessibility chicken embryos have been one of the preferred model organisms for developmental studies. Their disadvantage, the lack of genetic tools, could be overcome by the development of *in ovo* RNAi (*in ovo* RNA interference), a method that allows for temporal and spatial control of gene silencing *in vivo*.

KEYWORDS: *RNAi, chicken embryo,* in ovo *RNAi,* in utero *RNAi*, *nervous system, embryonic development*

INTRODUCTION

Methods of gene silencing are important tools with applications from basic research to drug development and therapy. Different needs in these fields of applications have brought forth different solutions. Due to the possibilities to block gene function in large-scale screens invertebrates were the model organisms of choice for the molecular analysis of physiological and developmental processes (Friedman and Perrimon, 2004). In so-called forward genetic screens mutagens were used to randomly mutate genomes of flies and worms. The resulting phenotypes of interest were selected and the genes containing the mutation causing these phenotypes were identified. Unfortunately, the elegance of these forward genetic screens cannot be transferred to reverse genetics in these organisms. It is much more difficult to cause a mutation in a specific target gene and then look at the consequences of this mutation. However, reverse genetics is required to study the role of genes in a given context or to study gene function in vertebrates, where forward genetic screens are largely restricted to zebrafish. Although there are attempts to apply forward genetics to mice the high cost and the requirement for large space will keep their numbers low (Carlson and Largaespada, 2005; Kile and Hilton, 2005). Many questions, for instance in organogenesis or neurobiology, cannot be studied in invertebrates and require the analysis of gene function in vertebrates. The mouse has become the model organism of choice for the majority of questions in basic and biomedical research. Mice are easy to breed and house in a lab environment. For many aspects of human physiology and disease there would be a better model than the mouse but none of them offers a comparable toolkit for gene manipulations. Because mice can be reconstituted from embryonic stem (ES) cells that can be manipulated in culture sophisticated manipulations of the mouse genome are possible (for a recent review see Glaser et al, 2005). Still, there are limitations in using the mouse as a model when it comes to developmental studies. Due to the inaccessibility of mammals during gestation oviparous animals, i.e. fish, reptiles, and birds, are much easier to use for experimental manipulations *in vivo*. Their embryonic development is very similar and directly comparable to mammals, at least for those animals that do not undergo metamorphosis. However, the big disadvantage of fish, reptiles, and birds as model systems is the lack of

genetic tools. RNAi is about to change that (Dykxhoorn and Lieberman, 2005). In fact, RNAi opens new possibilities of gene silencing in a temporally and spatially controlled manner that allows for studies that would be impossible with the available classical genetic tools. Spatial restriction can be achieved in mice by the use of sophisticated CreLox technologies and inducible promoters. These allow for the change in gene expression in the adult mouse without affecting gene function during development. However, there is still no way to control gene expression temporally in the precise manner that is required for studies of embryonic development. In this review we describe the advantages of RNAi technology for functional gene analysis during organogenesis using the nervous system as an example. We discuss the advantages and disadvantages of different model organisms in this context. Based on its easy accessibility during embryonic development the chicken is one of the preferred model organisms for developmental studies (Stern, 2005). The applicability of RNAi in chicken embryos made this model organism a perfect system to study gene function in a wide variety of tissues throughout development.

ANALYSIS OF GENE FUNCTION DURING DEVELOPMENT OF THE NERVOUS SYSTEM

The development of an organism requires a precise timing of gene expression in a spatially restricted manner. Many genes play a role in different tissues and during more than one time window. Excellent examples demonstrating this are morphogens (Tabata and Takei, 2004, see below). Morphogens are signaling molecules involved in very early aspects of development. They act in a concentration-dependent manner on responsive cells to induce their differentiation to a particular cell type (Ashe and Briscoe, 2006). Morphogens include members of the hedgehog, the Wnt, the Fgf, and the TGFβ family. More recently, new roles for morphogens during later stages of development have been discovered (Stoeckli, 2006; Charron and Tessier-Lavigne, 2005; Zou, 2004; Ciani and Salinas, 2003; Salinas, 2003). These studies have been possible thanks to sophisticated loss-of-function approaches in mice by restricting loss of gene function to a specific cell type (Charron et al, 2003) or by taking advantage of temporal control of gene silencing in the chicken embryo (Bourikas et al, 2005a).

Due to its complexity the nervous system takes a long time to develop. In fact in many species including humans neural development extends well beyond birth. It includes a variety of processes and genes that are involved in the development of other organs. For instance, genes involved in cell migration are often the same in the developing nervous system, in the heart or the vascular system (Carmeliet and Tessier-Lavigne, 2005). This has often hampered the analysis of genes by loss-of-function approaches in the nervous system, as mouse embryos died due to cardiac defects or defects in vasculogenesis and angiogenesis before their brains were fully developed. Alternatively, genes that have a function during several phases of embryonic development can only be studied during the earliest phase of activity, as their function during later stages is masked by aberrant initial development. Therefore, both classical reverse genetics as well as forward genetic screens have their limitations for the analysis of gene function during later stages of embryonic development.

MODEL ORGANISMS FOR DEVELOPMENTAL NEUROSCIENCE

Although forward genetic approaches in invertebrate animal models like *Drosophila melanogaster* and *Caenorhabditis elegans* contributed much to our understanding of neural development, for many questions vertebrate model systems are required (Anderson and Ingham, 2003). Depending on a developmental neuroscientist's demands, the vertebrate model organism of choice has to fulfil several criteria including availability of techniques for gene manipulation as well as easy accessibility during development. In addition, general requirements for the usefulness of a species as model organism have to be considered, such as the amount of money and time required for generating mutants, the availability in sufficient numbers and the easiness of husbandry of a model organism (Table 1).

The mouse: From conventional knockouts to *in utero* RNAi

The mouse is the most widely used model organism in developmental neuroscience, because techniques for loss- and gain-of-function approaches based on homologous recombination in ES cells are available (Carlson and Largaespada, 2005; Kile and Hilton, 2005). However, creating knockout animals by using homologous recombination in ES cells is still very time-consuming and cost intensive, and therefore its usefulness for the analysis of a large number of genes is limited. Furthermore, conventional gene knockout strategies may result in embryonic lethality precluding the analysis of gene function in the developing nervous system. As mentioned above, disrupting the expression of a gene of interest early in development prevents any further functional analysis at later stages because cell types or entire structures may not form (Chiang et al, 1996; Ihle, 2000).

To overcome problems of conventional knockouts, recombinase systems under the control of cell or tissue-specific promoters have been developed to allow conditional gene knockouts in mice (Gawlik and Quaggin, 2004). In addition to the widely used CreLoxP system, two other recombinase systems have been used successfully: the Flp-FRT system from Saccharomyces cerevisiae and the phiC31 integrase (Dymecki, 1996; Belteki et al, 2003). Although conditional knockout technology using recombinase systems have provided insight into neural development, these strategies are limited by the requirement of cell- or tissue-specific promoters (Zhu et al, 2001; Blaess et al, 2004; Lewis et al, 2004). Furthermore, two transgenic mouse lines are required to knockout one gene: One mouse line that derives the Cre recombinase under the control of the tissue-specific promoter, and the other expressing the target gene flanked by loxP sites. Some temporal control of gene expression in adult mice has been achieved with the development of tetracycline-sensitive and tamoxifen-inducible Cre recombinase systems (Lewandoski, 2001; Metzger and Chambon, 2001; Morozov et al, 2003). However, these systems do not al-

low short-term switches of gene expression that are required during embryonic development.

Additional problems with knockout mice are genetic redundancy. Other members of the family of the targeted gene can compensate for the loss of a gene's function to a degree that silencing one gene would not result in a detectable loss-of-function phenotype, hence requiring the generation of double- or triple knockout mice. The difficulties in generating conditional double or triple knockout mice would further complicate or prevent functional gene analysis during development.

RNA interference (RNAi), a conserved response to dsRNA resulting in specific gene silencing, represents an alternative way of blocking gene expression to conventional and conditional knockout technologies in mice (Lewis et al, 2002; McCaffrey et al, 2002; Prawitt et al, 2004). Hasuwa and colleagues showed long-term down-regulation of EGFP in variety of organs of adult transgenic mice with a transgene-based RNAi system (Hasuwa et al, 2002). They used the polymerase III promoter H1 to drive expression of an shRNA.

Adenovirus-mediated RNAi resulting in specific gene silencing in mouse brain has been established and offers the possibility for temporal control of gene silencing in adult mice (Xia et al, 2002). Because mouse embryos develop *in utero* they are not easily accessible during prenatal stages for *in vivo* manipulations. RNAi in post-implantation mouse embryos using electroporation has been developed to knockdown genes during embryonic development (Calegari et al, 2002). Thus, very short-term experiments are possible because culture procedures for mouse embryos have been developed (Calegari et al, 2004). However, the time window for these mouse embryo culture systems is restricted to two days and available only for embryos between E7 and E13. Therefore the embryonic stages that can be studied are very limited. In order to study long-term functions of genes involved in brain development *in utero* electroporation guided by ultrasound has been developed for mice and rats (Takahashi et al, 2002; Bai et al, 2003). In contrast to whole embryo cultures, the embryos electroporated *in utero* can be maintained and analyzed from early embryonic to postnatal or adult stages. *In utero* electroporation in combination with RNAi has been used for the functional characterization of doublecortin during cortical development (Bai et al, 2003). Low efficiency and the requirement for expensive equipment for *in utero* electroporation limit the wide applicability of this approach, as does the problem of low spatial resolution.

Table 1. Comparison of advantages and disadvantages of different loss-of-function approaches for developmental studies. The number of + or – signs indicates how much a particular issue is adding to the advantage (+) or disadvantage (–) of a particular approach. Obviously, the different approaches require technical expertise that cannot be taken into account for the comparison.

Technique	Costs	Labtime	Temporal control	Spatial control	Limitations
Conventional knock-outs (Mouse)	-	-	- -	- - -	No spatial and temporal control
Conditional knock-outs (Mouse)	- -	- -	-	+++	No temporal control Specific promoters required
Inducible knockout (Mouse)	- - -	- - -	++*	+++	Specific promoters required
Morpholinos (Zebrafish)	++	+++	-	-	
Virus-mediated RNAi (Mouse, Chicken)	-	+	++*	++	
RNAi: *In utero* electroporation of si/shRNA (Mouse, Rats)	+	+	++	-	Poor spatial resolution
In ovo RNAi (Chicken)	+++	+++	+++	+++	Embryonic stages only

*Temporal control is not available for embryonic stages

The Zebrafish as a model organism for developmental neuroscience

The zebrafish is a small tropical fish that represents an alternative vertebrate model organism to the mouse because of its rapid development *ex utero.* Embryos are translucent and therefore ideal for *in vivo* imaging. Improved methods for mutagenesis, transgenesis and gene targeting increase the usefulness of the zebrafish as a model organism for functional genomics (Ekker, 1999; Patton and Zon, 2001; Udvadia and Linney, 2003). Although chemical screens are highly effective in generating loss-of-function mutants, the process of identifying the mutated gene is laborious (Zhang et al, 1998; Talbot and Schier, 1999). Furthermore, as mentioned above, forward genetic approaches may not be useful for specific questions and do not allow for spatiotemporal control of gene silencing.

Antisense technology is a useful tool for specific gene silencing during development and has been applied in many species (Audic et al, 2001; Coonrod et al, 2001; Howard et al, 2001; Yang et al, 2001; Kos et al, 2003). Chemical modification of oligonucleotides has improved their stability and therefore increased their applicability *in vivo*. Morpholino phosphorodiamidate oligonucleotide-mediated gene inactivation is widely used for the analysis of gene function in zebrafish (Summerton and Weller, 1997; Nasevicius and Ekker, 2000; Corey and Abrams, 2001; Heasman, 2002; Sumanas and Larson, 2002). Morpholinos show a lower cellular toxicity and fewer side effects compared to conventional antisense nucleotides (Pickart et al, 2004). Usually morpholinos are microinjected into zebrafish embryos between the one- and the eight-cell stage. For effective gene inactivation the morpholino has to be complementary to the 5'UTR or the translation initiation site (Summerton and Weller, 1997; Heasman, 2002). The degree of gene silencing depends on the injected morpholino concentration and the extent of dilution due to cell proliferation (Heasman, 2002). Because morpholinos have to be injected into zebrafish embryos at very early developmental stages they loose effectiveness after a few days. Thus, functional analysis of genes expressed at later developmental stages cannot be achieved by this approach. The use of high concentrations of morpholinos increases the risk of inducing non-specific and toxic effects including cell death and neural degeneration (Nasevicius and Ekker, 2000; Braat et al, 2001; Karlen and Rebagliati, 2001; Lele et al, 2001). Lipofection can be used to improve cellular penetration of antisense oligonucleotides *in vitro* as well as *in vivo* (Juliano et al, 1999; Stenkamp et al, 2000). However, lipofection is associated with toxicity *in vitro* and even more importantly *in vivo.*

***In ovo* RNAi - a tool for functional gene analysis in chicken embryos allows for temporal control of gene silencing**

For a long time the chicken embryo was a classical model organism for developmental studies in vertebrates because of its easy accessibility during development (Bourikas and Stoeckli, 2003; Bourikas et al, 2005b; Stern, 2005). *In ovo* as well as *ex ovo* culture methods of chicken embryos offer the possibility for *in vivo* manipulations throughout embryonic development (Perry, 1988; Stoeckli, 2003; Krull, 2004; Luo and Redies, 2005). After 21 days, at the time of hatching, the nervous system is fully developed and functional. The chicken genome is sequenced, and thus, comparisons of chicken genes with the human and the mouse or rat genomes are very easy (Hillier et al, 2004).

Due to biological constraints and the lack of ES cells it is not possible to manipulate the chicken genome with the same toolkit that is available for the mouse. The technology to generate transgenic chickens has been developed very recently (Mozdziak et al, 2003; Chapman et al, 2005), but the size and the long generation time of chickens limit the feasibility to breed them in a lab animal facility.

The easy accessibility of the chicken embryo during development of the nervous system was exploited for functional studies at the protein level using function-blocking antibodies (Stoeckli and Landmesser, 1995; Stoeckli et al, 1997; Burstyn-Cohen et al, 1999; Perrin et al, 2001). However, the limited availability of function-blocking antibodies severely restricted the usefulness of this approach. As an alternative, viral vector-based expression systems were developed to express dominant-negative proteins (Morgan and Fekete, 1996; Logan and Tabin, 1998). Morpholinos were also used successfully in chicken embryos (Kos et al, 2003; Tucker, 2004), although they may be more difficult to target to specific tissues than regular oligonucleotides (Krull, 2004). More recently, *in ovo* electroporation as an efficient method of gene transfer in chicken embryos for the temporally and spatially controlled ectopic expression of a gene of interest was established (Table 2; Muramatsu et al, 1997; Momose et al, 1999; Nakamura and Funahashi, 2001; reviewed in Bourikas and Stoeckli, 2003). Because loss-of-function phenotypes are usually more informative than gain-of-function phenotypes for the functional characterization of a gene of interest (Hudson et al, 2002) both viral vector-mediated and electroporation-based gene transfer depended on the availability of a dominant-negative mutant of the gene of interest.

To overcome the limitations of the chicken embryo as a model system for functional gene analysis, *in ovo* RNAi, a combination of *in ovo* electroporation for efficient nucleic acid transfer and RNAi for specific gene silencing has been established (Figure 1; Pekarik et al, 2003; Stoeckli, 2003; Bron et al, 2004; Krull, 2004).

Gene silencing has been achieved with several approaches and in many different areas of the nervous system (Table 2) but also other tissues (e.g. Toyofuku et al, 2004). The parameters for electroporation have to be adapted to the age of the embryo and the target tissue (Itasaki et al, 1999; Krull, 2004; Luo and Redies, 2004). More important than the electroporator (see Krull, 2004, for a comparison of different brands) is the choice of electrodes. For a focal transfer of nucleic acids a needle electrode is often placed directly into the tissue (Oberg et al, 2002; Luo and Redies, 2004). For a more widespread transfer a non-invasive method with wire or platelet electrodes is chosen (Dai et al, 2005; Nakamura et al, 2004; Toyofuku et al, 2004; Matsuda and Cepko, 2004). Some researchers found sonoporation more effective than electroporation for nucleic acid transfer into mesenchymal tissue (Ohta et al, 2003, but see Swartz et al, 2001a; Eberhart et al, 2004).

Table 2. *In ovo* electroporation or RNAi has been successfully used to change gene expression in chicken embryos in a temporally and spatially controlled manner.

Target tissue	Loss of function by			Gain of function	Reference
	dsRNA[1]	siRNA	shRNA		
Neural tube			x		Chesnutt and Niswander, 2004 Dai et al, 2005
		x	x		Bron et al, 2004
	x	x	x		Rao et al, 2004
	x				Pekarik et al, 2003 Bourikas et al, 2005a Stepanek et al, 2005
				x	Luo and Redies, 2005
Cranial neural tube			x		Katahira and Nakamura, 2003
		x			Nakamura et al, 2004
Cerebellum				x	Luo and Redies, 2004 and 2005
Tectum			x		Yamagata and Sanes, 2005
Retina, lens				x	Chen et al, 2004
Limbs, mesenchyme				x[2]	Swartz et al, 2001a,b Eberhart et al, 2002 Oberg et al, 2002; Krull, 2004
Somites				x	Swartz et al, 2001a, b Eberhart et al, 2002 Scaal et al, 2004
Heart		x			Toyofuku et al, 2004

Note: Detailed protocols can be found in: Stoeckli, 2003; Krull, 2004; Sato et al, 2004
[1] dsRNA refers to the use of long fragments of dsRNA (200 – 2000 bp)
[2] In some studies dominant-negative proteins were expressed to get loss-of-function phenotypes

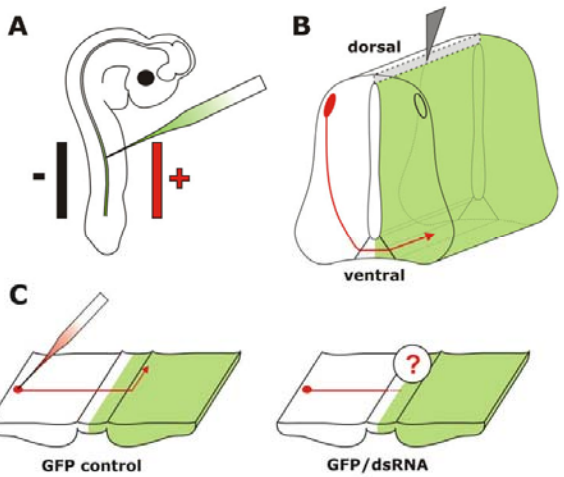

Figure 1. *In ovo* RNAi is an efficient method to silence genes in a temporally and spatially controlled manner in the developing neural tube. The chicken embryo can be accessed through a window cut into the eggshell. Phosphate-buffered saline containing long dsRNA, siRNA, or a plasmid encoding shRNA and 0.04% Trypan Blue is injected into the neural tube of the developing embryo with a glass pipette (**A**). In order to visualize the area where the injected RNA was taken up or as a control a plasmid encoding GFP can be co-injected. Wire electrodes are positioned parallel to the longitudinal axis of the embryo. Due to the negative charge of the

nucleic acids cells toward the side of the anode are transfected. On average, we achieved 60% transfection efficiency in the targeted area of the neural tube of a 3-day-old embryo using 5 pulses of 25 Volts and of 50 msec duration (Pekarik et al, 2003). We use a one second inter-pulse interval. Depending on the position of the electrodes the target area can be selected. Positioning the electrodes dorsally will only target dorsal cell of the developing neural tube, whereas a more ventral position will result in transfected cells all along the dorso-ventral axis (as shown in **B**). To analyze the resulting phenotypes induced by knockdown of the target gene a variety of methods can be used. As an example we illustrate visualizing the trajectory of dorsolateral commissural neurons. These neurons extend their axons toward the floor plate, the structure that forms the ventral midline of the spinal cord. Axons cross the ventral midline before turning rostrally along the contralateral side of the floor plate (Bourikas et al, 2005a). The trajectory of these axons can be visualized by application of the lipophilic dye DiI to the cell bodies of commissural neurons (**C**). The comparison between control embryos, injected with a plasmid encoding GFP only or a control siRNA, with embryos injected with the target-specific dsRNA would reveal phenotypes in axon pathfinding. In the situation shown here, these would be caused by cell non-autonomous functions of the target gene as commissural axons from the side contralateral to the electroporated area are traced. For detailed protocols see references in Table 2.

In contrast to mammalian cell lines and non-embryonic tissue long dsRNA can be used in chicken embryos without induction of unspecific effects. No general inhibition of protein synthesis or induction of apoptosis has been observed (Pekarik et al, 2003; Chesnutt and Niswander, 2004). Similarly, no unspecific effects were seen in mouse oocytes (Stein et al, 2005) and cell lines derived from embryonic tissue (Billy et al, 2001). To avoid unspecific effects in postnatal mice and cell lines short interfering RNAs (siRNAs) can be used (Caplen et al, 2001; Elbashir et al, 2001). They have been successfully used in chicken embryos as well (Katahira and Nakamura, 2003; Sato et al, 2004).

As electroporation does not affect 100% of the cells in the target area (Pekarik et al, 2003; Luo and Redies, 2005) it may be important to identify those cells that did take up the siRNA or the dsRNA. In many cases co-electroporation of a plasmid encoding EGFP has been chosen and was sufficient (Pekarik et al, 2003; Nakamura et al, 2004). However, for an unequivocal identification of transfected cells the use of a vector-based approach has been developed. Driven by a polymerase III promoter short-hairpin RNAs (shRNAs) are produced in the cell. Because the plasmid contains an IRES site and also encodes EGFP all transfected cells are easily identified. The commonly used pol III promoters H1 and U6 were found to work well in chicken embryos (Katahira and Nakamura, 2003; Chesnutt and Niswander, 2004; Bron et al, 2004; Dai et al, 2005).

No matter whether long dsRNA, siRNAs, or shRNAs are used, the knockdown of target genes has been found to be efficient. Obviously, RNAi only prevents the synthesis of new protein. It cannot remove the pre-existing protein from a cell. Thus, for most effective gene silencing the injection and electroporation has to be carried out before the onset of gene expression. Gene silencing by RNAi was found to be long lasting in non-proliferating cells (from at least 9 days to 3 weeks; Sato et al, 2004; Omi et al, 2004). In cell culture or in tissues where cells proliferate the effect is diluted with successive cell divisions and usually decreases after 3-5 days. The use of a mixture of 3-5 different siRNAs is generally considered to be more effective than the use of a single siRNA. For that purpose mixtures of siRNAs can be generated *in vitro* from long dsRNAs by digestion with RNase ONE (Rao et al, 2004). The production of siRNAs in situ from vectors encoding shRNAs was found to extend the length of gene silencing compared to siRNAs (Bron et al, 2004).

As mentioned above, it is not necessary to generate the mixture of siRNAs *in vitro* before injection as long as embryonic tissue is used. Long dsRNA was always effective in gene silencing in our hands, presumably because they always give rise to a mixture of siRNAs that contains many effective ones. If siRNAs are designed with algorithms that are either freely available (Ui-Tei et al, 2004; Nakamura et al, 2004) or commercially used by companies selling siRNAs, it can still be frustrating to find effective ones.

Concerns about so-called off-target effects, i.e. the silencing of one or several non-target genes due to full or partial sequence homology with the siRNA have been raised (reviewed in Jackson and Linsley, 2004). Obviously such an event cannot be fully excluded but there are some rules to minimize the risk of off-target effects (Qiu et al, 2005). Firstly, it is of course essential to carry out proper BLAST analyses and to avoid sequences that are found in genes other than the target gene. Secondly, more than one (mixture of) siRNA or long dsRNA fragment should be used independently, as it is unlikely that they would have the same off-target effect, i.e. silence the same non-target genes. Thirdly, the concentration of the siRNA should be as low as possible. Generally, unspecific effects are not expected when concentrations are 20 nM or lower. When using long dsRNA we routinely get effective silencing with dsRNA concentrations in the range of 0.1-1 nM.

It is difficult to compare the efficiency of gene silencing between different RNAi approaches in the absence of systematic studies. The percentage of gene knockdown correlates with the concentration of siRNA, or dsRNA, respectively. Rao and colleagues compared the efficiency of siRNAs with a mixture of siRNAs produced *in vitro* from long dsRNA by RNase ONE, and long dsRNA (Rao et al, 2004). They concluded that siRNAs were more effective than the mixture of siRNAs created *in vitro* and long dsRNA, respectively. However, this conclusion is flawed by the fact that the number of effective siRNAs created from long dsRNA *in vivo* or *in vitro* by RNase ONE (where also ineffective siRNAs shorter than 21 bp are generated) is unknown. They used the same amount of siRNAs (200 ng/µl) and long dsRNA (700 bp fragment). Therefore, the concentration of the long dsRNA was roughly 30fold lower than the concentration of siRNAs. The effect of the much lower concentration of long dsRNA was still more than half as efficient as the siRNAs (53% compared to 90%), thus raising some doubts, whether siRNAs are really more efficient than long dsRNA.

The major advantages of *in ovo* RNAi are the temporal and spatial control of gene expression. This is due to the accessibility of the chicken embryo *in ovo* and the possibility to culture embryos 'shell-less' in dishes (Perry, 1988; Luo and Redies, 2005). Furthermore, it is easy to knockdown more than one gene at the same time. Full-length cloning is not required, as cDNA fragments or ESTs can directly be used to produce dsRNA by *in vitro* transcription. Therefore, *in ovo* RNAi represents a fast and inexpensive tool for functional genomics. It can easily be adapted for the assessment of different developmental processes (Table 2; Bourikas and Stoeckli, 2003; Krull, 2004; Eberhart et al, 2004; Toyofuku et al, 2004; Luo and Redies, 2004 and 2005; Luo et al, 2004).

Taking advantage of the major asset of *in ovo* RNAi, i.e. precise temporal control over gene silencing during embryonic development, a role for the morphogen sonic hedgehog (SHH) in postcommissural axon guidance could recently be demonstrated (Bourikas et al, 2005a). During early stages of embryonic development SHH is involved in inductive and patterning processes including control of left-right asymmetry and formation of the limb (reviewed by Marti and Bovolenta, 2002; Jacob and Briscoe, 2003). Slightly later in development, Shh was shown to act in parallel to netrin-1 as a chemoattractant for dorsal commissural axons (Charron et al, 2003). All these effects of Shh are mediated by a receptor complex composed of Patched and Smoothened. Interestingly, these receptors are not involved in Shh's effect on postcommissural axons. After crossing the floor plate, the ventral midline of the spinal cord, commissural axons are no longer expressing Patched and Smoothened. The repulsive effect of Shh on postcommissural axons is mediated by Hip (Hedgehog interacting protein). Thus, commissural axons switch from being attracted by Shh before midline crossing to being repelled by a concomitant switch in receptor expression (Figure 2; Bourikas et al, 2005a; reviewed by Charron and Tessier-Lavigne, 2005). The analysis of this rapid change in responsiveness to Shh would not have been possible without a method that allows for precise temporal control of gene silencing.

The large number of papers describing various approaches of RNAi-based gene silencing in chicken embryos demonstrates the versatility of the chicken embryo on the one hand and RNAi on the other hand. We have only been able to include some of the studies carried out in the last 2 to 3 years and focused largely on the development of the nervous system. The multitude of approaches, siRNAs versus shRNAs or long dsRNA, different electrodes used with different electroporation parameters may be confusing at first glance. However, transfection of a plasmid encoding GFP is an easy way to get started. It allows for fast assessment of transfection efficiency in the target area and for selection of experimental parameters. Above all, it provides a fast method to test and train the skills of the experimenter to handle chicken embryos *in ovo* or *ex ovo*. Beginners should use GFP expression to assess their skills with respect to reproducibility of electroporation and the absence of artefacts due to tissue damage caused by injection or by touching embryonic tissue with the electrodes. The fact that both *in ovo* RNAi and *ex ovo* RNAi require some manual skills for handling live embryos may in fact represent their biggest disadvantage. The best way to learn handling chicken embryos is by visiting a lab where they are routinely used for research.

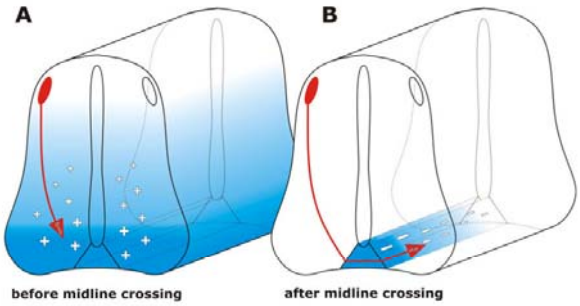

Figure 2. *In ovo* RNAi allows for temporal control of gene silencing in the developing neural tube. In order to study gene function during embryonic development precise temporal and spatial control of gene silencing is required. Classical knock-out strategies do not allow for functional gene analysis during later stages of development, as lack of target gene expression during the first time window would preclude the analysis of its function during later stages. An example illustrating the requirement for temporal control is the analysis of SONIC HEDGEHOG (SHH). Shh is a morphogen that is required for differentiation of cells in the spinal cord during early stages of development (Jessell, 2000). Slightly later, Shh acts in parallel to Netrin-1 as a long-range guidance cue, attracting dorsal commissural axons toward the floor plate (**A**; Charron et al, 2003). This attractive effect of Shh is mediated by the co-receptor formed by Patched and Smoothened (Smo). A few hours later, after commissural axons have crossed the floor plate, Shh acts as a repulsive guidance cue, directing post-commissural axons rostrally (**B**; Bourikas et al, 2005a). The repulsive activity of Shh is mediated by Hedgehog-interacting protein (Hip). Thus, within a short period of time, commissural axons switch receptors (from Smo to Hip) that allow them to respond differently to Shh gradients.

CONCLUSIONS

The development of *in ovo* RNAi has not only reinstated the importance of the chicken embryo as a model organism for developmental studies but it has added precise temporal control of gene silencing to our toolkit for gene manipulations. As demonstrated in recent studies, temporal and spatial control of gene function is an important aspect of functional gene analysis during embryonic development.

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STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

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1.5 Aims of the thesis

The aims of my PhD thesis were:

• Establishment of *ex ovo* electroporation and *ex ovo* RNA interference (RNAi) as novel tools for functional gene analysis in the developing chicken cerebellum (**Chapter 2**)
• The characterization of Axonin-1 as a guidance cue in parallel fiber extension in the developing cerebellum of the chicken embryo (**Chapter 2**)
• Identification and functional characterization of Endoglycan during commissural axon pathfinding and radial Purkinje cell migration in the chicken embryo (**Chapter 3**)

2. Axonin-1/TAG-1 is required for pathfinding of granule cell axons in the developing cerebellum

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key words: chicken embryo, *ex ovo* RNAi, gene silencing, granule cell development, parallel fibers, axon guidance

(submitted to BMC Neural Development)

2.1 Abstract

Background:

Neural development consists of a series of steps, including neurogenesis, patterning, cell migration, axon guidance, and finally, synaptogenesis. Because all these steps proceed in a constantly changing environment, functional gene analyses during development have to take time into account. This is quite challenging, however, as loss-of-function approaches based on classical genetic tools do not allow for the precise temporal control that is required for developmental studies. Gene silencing by RNAi in combination with the chicken embryo or with cultured embryos opens new possibilities for functional gene analysis *in vivo*.

Axonin-1/TAG-1 is a cell adhesion molecule of the immunoglobulin superfamily with a well defined temporal and spatial expression pattern in the developing vertebrate nervous system. Axonin-1/TAG-1 was shown to promote neurite outgrowth *in vitro* and to be required for commissural and sensory axon pathfinding *in vivo*.

Results:

To knock-down Axonin-1 in a temporally and spatially controlled manner during development of the nervous system, we have combined RNAi with the accessibility of the chicken embryo even at late stages of development. Using *ex ovo* RNAi, we analyzed the function of Axonin-1/TAG-1 in cerebellar development. Axonin-1 is expressed in postmitotic granule cells while they extend their processes, the parallel fibers. In the absence of Axonin-1 these processes were still extending but no longer in a parallel manner to each other or to the pial surface of the cerebellum.

Conclusion:

Axonin-1/TAG-1 is required for the navigation but not for the elongation of granule cell processes in the developing cerebellum *in vivo*.

2.2 Introduction

Axonin-1/TAG-1 is a cell adhesion molecule of the immunoglobulin superfamily that was shown to be an axon guidance cue in the central nervous system *in vivo* [1,2]. Commissural axons in the spinal cord require Axonin-1 for midline crossing [3]. In the absence of interactions between growth cone Axonin-1 and floor-plate NrCAM the floor plate is perceived as repulsive and growth cones fail to enter [4]. Sensory neurons from dorsal root ganglia depend on Axonin-1 for subpopulation-specific navigation to the gray matter [5]. In the absence of Axonin-1 function, nociceptive fibers failed to innervate their target layers in the dorsal spinal cord and extended into areas normally innervated by mechanoreceptive fibers. In the cerebellum, Axonin-1 is expressed in postmitotic granule cells at the time when they extend their processes, the parallel fibers [6,7].

The cerebellum is responsible for motor coordination but is also involved in cognitive processes [8,9]. Malformations or damage to the cerebellum have been linked to several human disabilities, including ataxia, cerebral palsy, and epilepsy [8]. In line with the more recent literature describing a contribution of the cerebellum to cognitive processes, changes in cerebellar structure and function have been linked to mental retardation, autism, and schizophrenia [9]. The development of the cerebellum has been studied in mouse [8,10-13] and in chicken embryos [14-18]. Cells of the cerebellum originate from rhombomere 1, the anterior-most part of the hindbrain [18]. With one notable exception, the granule cells, all cells are born in the ventricular zone and migrate radially to reach their final destination in the mature cerebellum [11,13]. The rhombic lip, the contact site between the roof plate of the fourth ventricle and the neural tube gives rise to precursors of granule cells. These migrate tangentially on the pial surface of the cerebellar anlage to form the external germinal layer (EGL) [18-21]. After proliferation in the outer EGL, granule cell precursors differentiate in the inner EGL and extend their axons, the parallel fibers. Together, parallel fibers and dendrites of Purkinje cells form the molecular layer (ML). After the initial formation of parallel fibers, granule cells migrate radially through the Purkinje cell layer (PCL) to form the inner granule cell layer (IGL) [11]. Thus, the only cell bodies that remain in the molecular layer are interneurons, stellate and basket cells that originate from the ventricular zone [22, 23]. Although these processes are known at the cellular level, the molecular mechanisms regulating these distinct steps are poorly understood.

To test for a role of Axonin-1 in parallel fiber extension in the developing cerebellum we used *ex ovo* RNAi, a combination of RNAi and *ex ovo* culturing of chicken embryos. In contrast to our previously established method, *in ovo* RNAi [24], *ex ovo* RNAi enhances the accessibility of chicken embryos for loss-of-function approaches at late stages of development.
We developed *in ovo* RNAi as a tool to study gene function in a temporally controlled manner during development of the chicken nervous system [24-26]. Using this technique, we demonstrated for instance additional functions of the morphogen Sonic hedgehog (Shh) beyond patterning and differentiation [27]. During early stages of development Shh acts as a ventralizing factor in spinal cord patterning [28]. Later, still using Patched and Smoothened to mediate its effect, Shh attracts

commissural axons toward the floor plate [29]. Only a few hours later, Shh switches receptors and mediated by Hip (Hedgehog-interacting protein) acts as a guidance cue for postcommissural axons [30]. The analysis of Shh's function clearly demonstrates the importance of tight temporal control of gene silencing during embryonic development [25,26].

To extend the usefulness of the chicken embryo as a model organism to study later stages of neural development during which the embryo is no longer easily accessible in the egg, we established *ex ovo* RNAi. Chicken embryos can be transferred from the egg to a plastic dish without detrimental effects on their development. The absence of the eggshell enhances access to the developing embryo for experimental manipulation. *Ex ovo* RNAi, the combination of RNAi and *ex ovo* culturing of chicken embryos allowed us to study the role of Axonin-1 in parallel fiber formation. In the absence of Axonin-1 function neurites of granule cells still extended but failed to navigate correctly in the developing molecular layer. Rather than extending in a parallel manner, they invaded the layer of proliferating granule cells at the pial surface of the cerebellum.

2.3 Results

2.3.1 Embryos grown *ex ovo* can easily be accessed for manipulations of gene expression in a temporally and spatially controlled manner

To enhance its accessibility during later stages of development the chicken embryo can be cultured in a dish without adverse effects on development [31-34]. To have access to the developing cerebellum, we transferred embryos to a domed dish after the second day of incubation (Figure 2.1). As described previously [34] the transfer should be done no later than E2.5 (embryonic day 2.5) for best survival of the embryo. The survival rate of embryos depended on several factors, including temperature and humidity in the incubator, as well as the time of embryo transfer. Routinely, we found 62% of the embryos alive on E8, the time of injection in this study.

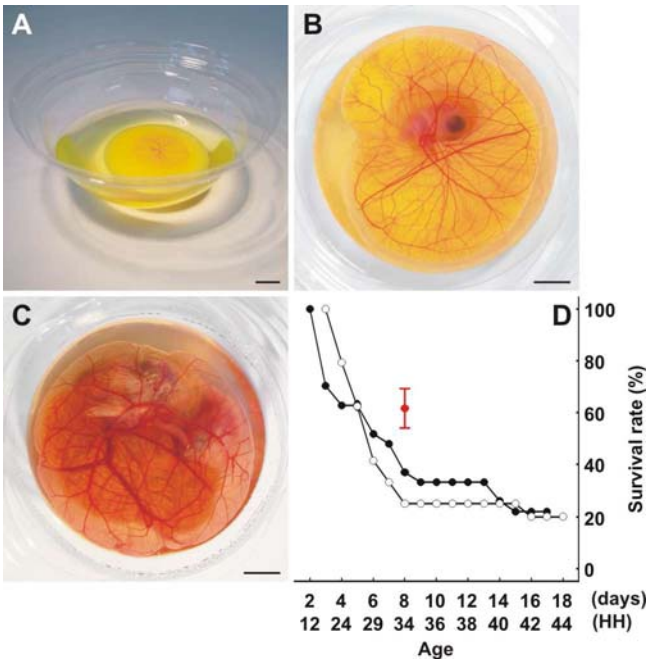

Figure 2.1 Chicken embryos can be cultured *ex ovo* to make them easily accessible for manipulations throughout embryonic development. After two days of incubation at 38.5°C the whole egg content was carefully transferred into a domed dish **(A)**. After 6 days of *ex ovo* culturing the embryos reached HH34 **(B)**, the time point when injections and electroporations were performed in this study. The chicken embryo can be kept alive throughout embryonic development. Due to an increasing number of blood vessels in the extraembryonic membranes at later developmental stages (as seen in **(C)** at E18), injections and electroporations become more difficult however. Depending on the time of transfer the survival rate varied slightly but not significantly. When the embryo was transferred after two days (black dots in **D**) the survival rate of the embryos decreased more slowly during the first eight days compared to embryos that were transferred after 3 days (white dots in **D**). In both cases the survival rates stabilized for the following days (after E8). Routinely we found 62% of the cultured embryos alive on E8 (red dot in **D**). The age of the embryos is indicated in days and corresponding developmental stages according to Hamburger and Hamilton [35]. Bars: 1cm.

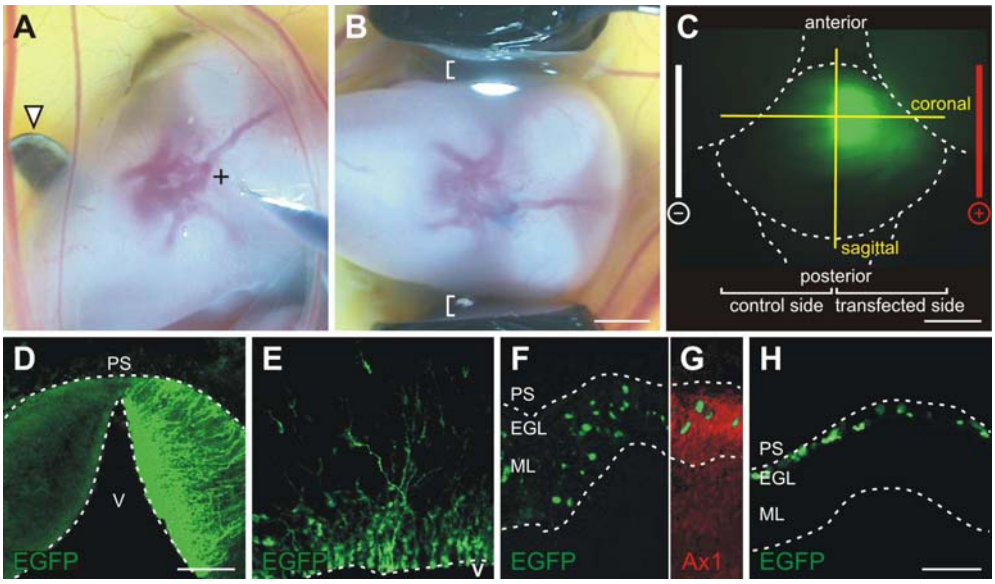

Figure 2.2 Different cerebellar layers can be targeted by *ex ovo* electroporation. The cerebellar anlage is positioned right under the bifurcation of the middorsal sinus and the middle cerebral vein. The injection site is indicated by + in (**A**). The head of the embryo was turned and stabilized with a hook prepared from a spatula (white arrowhead in **A**). Tweezer electrodes were placed parallel to the head (**B**). To prevent tissue damage it was important to avoid contact between embryo and electrode (indicated by white brackets in **B**). One half of the cerebellum was successfully transfected by electroporation (**C,D**). The untransfected half of the cerebellum could serve as an internal control for the analysis of phenotypes. Successful transfection of one half of the cerebellum after *ex ovo* electroporation using platelet electrodes is shown in a 250-µm-thick coronal slice (**D**). Depending on the depth of the injection different cerebellar layers could be targeted: Deep injections into the ventricle resulted predominantly in transfection of the ventricular zone labeling all cell types proliferating and migrating from there, such as Purkinje cells, interneurons, and glia cells (**E**). With superficial injections the developing molecular layer (**F,G**) and granule cells of the external germinal layer (**H**) were transfected. Parallel fibers in the developing molecular layer were visualized using Axonin-1 as a marker in **G**. PS Pial surface; V Ventricle; EGL external germinal layer; ML molecular layer. Bars: 2 mm in **A** and **B**, 1 mm in **C**, 500 µm in **D**, 100 µm in **E-H.**

Injections into the cerebellum had to be carried out without direct visual control. However, the blood vessels that are readily visible through the skin could be used as landmarks (Figure 2.2A). Using an expression plasmid encoding EGFP the proper injection site, the required depth of the injection as well as the best electroporation settings were determined. For electroporations we placed the head of the embryo between tweezer electrodes. To prevent tissue damage direct contact between embryo and electrodes was carefully avoided (Figure 2.2B). The cerebellum was efficiently transfected with 6 pulses of 99 ms duration at 40 V, as judged by the number of EGFP-expressing cells (Figure 2.2D-H). Only one half of the cerebellum expressed the transgene, because the negatively charged nucleic acids were migrating towards the anode (Figure 2.2C,D). Therefore, the untransfected side of the cerebellum could be used as a control for the analysis of potential phenotypes. When injections were

carried out at HH34 (stage 34, [35], E8), the survival rate one day after the experiment was 72%. However, the time point of the injection is not restricted to HH34. Injections and electroporations were performed successfully until HH36 (E10; not shown).

Depending on the depth of the injection, different cerebellar layers could be targeted. Injections into the ventricle resulted in transfection of the ventricular zone, the origin of Purkinje cells and interneurons (Figure 2.2E). After superficial injections into the cerebellar anlage cells of the developing molecular layer (ML; Figure 2.2F,G) and the EGL were transfected (Figure 2.2H) similar to observations made in postnatal rat pubs [36]. To get efficient transfection of all cerebellar layers the glass capillary was inserted into the ventricle and the injection pressure was maintained during its retraction.

2.3.2 *Ex ovo* electroporation and *ex ovo* RNAi did not induce apoptosis or morphological changes

To make sure that the injection of plasmids encoding EGFP or long dsRNA into the cerebellum and subsequent *ex ovo* electroporation did not induce non-specific effects, we checked for apoptosis and histological changes in the transfected area of the cerebellum (Figure 2.3). Apoptosis was compared between untreated control embryos (Figure 2.3A), control embryos injected and electroporated with the EGFP plasmid alone (Figure 2.3B), and experimental embryos injected and electroporated with EGFP and dsRNA derived from axonin-1 (Figure 2.3C). We did not find any difference in apoptosis between control and experimental embryos, indicating that our experimental procedure did not induce cell death. As demonstrated previously the use of long dsRNA did not induce unspecific effects in chicken embryos [24].

The comparison of sections from control-injected/electroporated and untreated control embryos stained with methylene blue confirmed the absence of morphological changes in experimental embryos (not shown). The cerebellar foliation and the organization of the EGL were unchanged 3 days after electroporation. Furthermore, the organization of Purkinje cells and Bergmann glia cells was visualized by staining for Calbindin (Figure 2.3D,E,F) and Vimentin (Figure 2.3G,H,I), respectively. No changes were observed when experimental embryos were compared with control embryos 4 days after electroporation (HH38). Taken together, we did not find any evidence that *ex ovo* electroporation induced apoptosis, morphological changes, or developmental delays during cerebellar development.

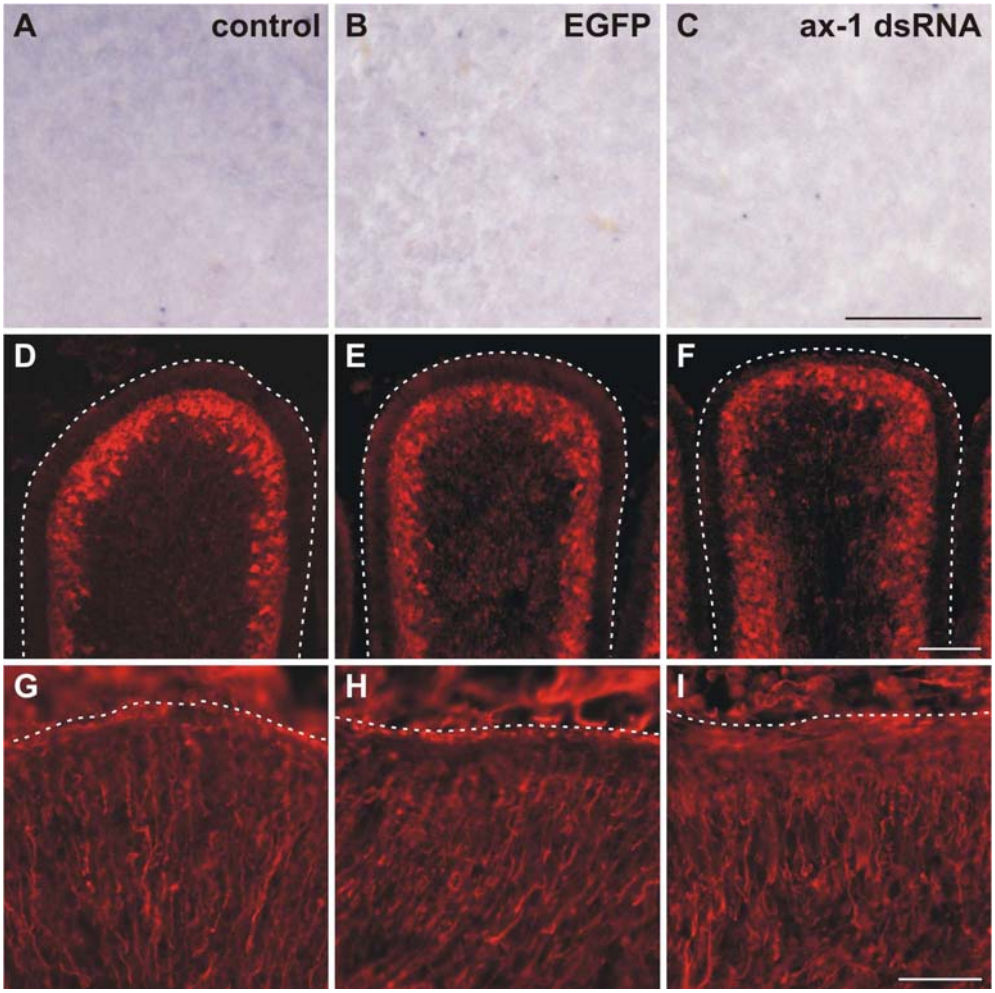

Figure 2.3 *Ex ovo* electroporation and RNAi using long dsRNA does not induce apoptosis and does not disturb cerebellar organization. Apoptosis was investigated by TUNEL in 30-µm-thick sagittal sections one day after electroporation. Corresponding areas are shown in **A-C**. No difference in apoptosis was detected between untreated embryos (**A**), embryos injected with EGFP plasmids alone (**B**) and embryos injected with axonin-1 dsRNA (**C**). The organization and the development of Purkinje cells analyzed by Calbindin staining (**D-F**) did not differ between untreated control embryos (**D**), EGFP-expressing control embryos (**E**), and embryos electroporated with axonin-1 dsRNA (**F**). Similarly, no changes in Bergmann glia cells were detectable after staining for Vimentin (**G-I**), when untreated (**G**), EGFP-expressing control embryos (**H**), and embryos lacking Axonin-1 (**I**) were compared. Bars: 100 µm in **A-C**, 200 µm in **D-F,** and 50 µm in **G-I**.

2.3.3 *Ex ovo* RNAi effectively silences the targeted gene

Axonin-1 is expressed in postmitotic granule cells in the EGL of the developing cerebellum ([6,7]; this study). To demonstrate efficient downregulation of Axonin-1 in these cells by *ex ovo* RNAi we mixed dsRNA derived from axonin-1 with a plasmid encoding EGFP with a molecular ratio of 50:1. EGFP expression was used to identify the position and the size of the electroporated area in the cerebellum. Electroporation of axonin-1 dsRNA efficiently knocked down Axonin-1 protein (Figure 2.4). Caudal cerebellar sections taken from an experimental embryo exhibited a patchy pattern of Axonin-1

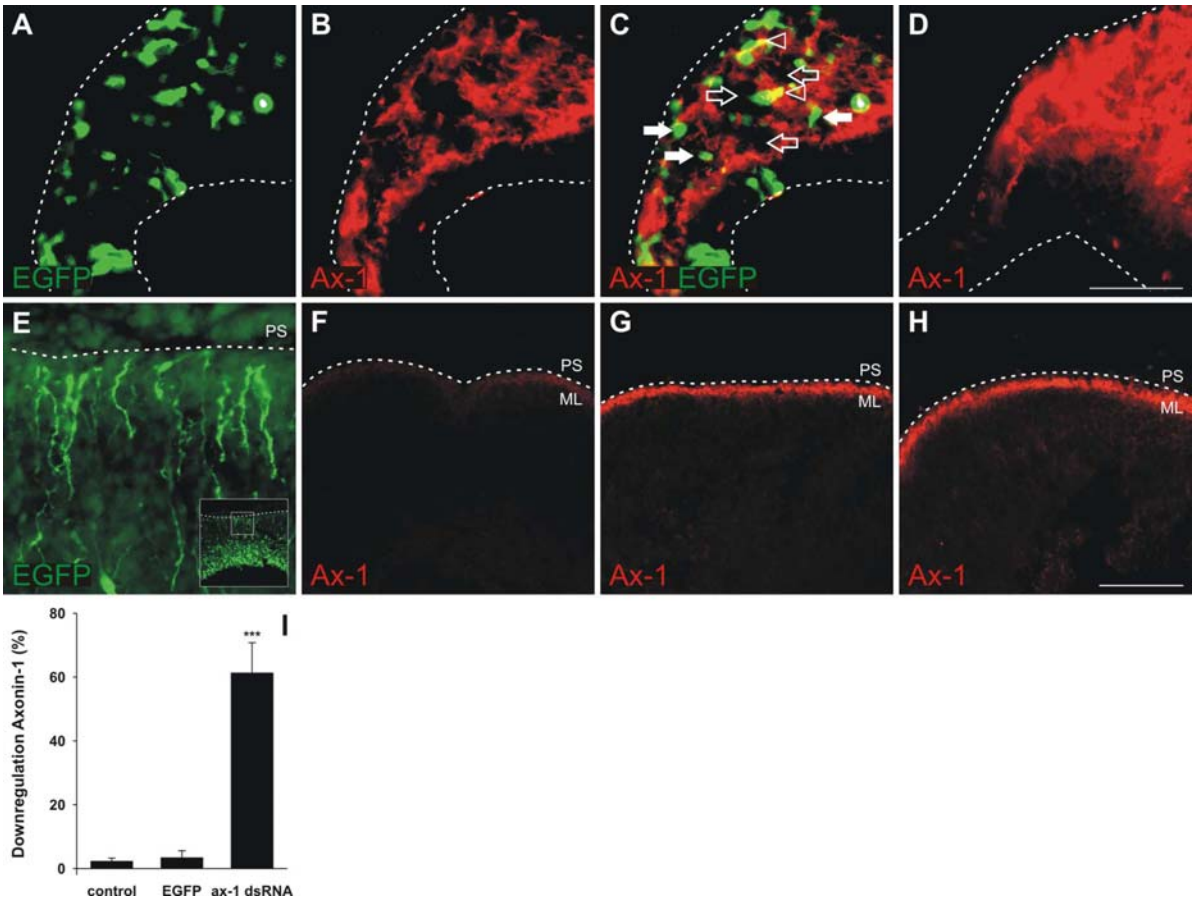

Figure 2.4 Gene silencing by *ex ovo* RNAi is efficient. The efficiency of Axonin-1 downregulation was demonstrated in caudal sections taken from HH35 cerebella one day after injection and electroporation of dsRNA derived from axonin-1 mixed with a plasmid encoding EGFP (50:1). EGFP expression was used to identify the electroporated area of the cerebellum (**A**). Expression of Axonin-1 was lost from many cells after *ex ovo* RNAi resulting in a patchy appearance of the granule cell layer (**B**, open arrows in **C**). In corresponding sections of a control embryo Axonin-1 staining appeared to be homogenous (**D**). The number of EGFP-expressing cells (white arrows in **C**) was much smaller than the number of cells that failed to express Axonin-1 due to the molecular ration of 50:1 for dsRNA and EGFP plasmid. Very few cells were yellow indicating both EGFP and Axonin-1 expression (arrowheads, **C**). For quantitative analysis 30-µm-thick sagittal sections were stained for Axonin-1. EGFP expression was used to identify the electroporated area of the cerebellum (**E**). Staining intensities were compared between experimental (**E,F**) and control (EGFP) embryos (**H**) one day after electroporation. On average the injection and electroporation of axonin-1 dsRNA reduced the Axonin-1 protein level in the transfected area by 61.3±9.4% (**F** and **I**; n = 4 embryos; $p<0.0001$ for comparison with both control groups). As expected, there was no change in Axonin-1 protein levels in the untransfected part of the cerebellum of embryos treated with dsRNA (**G**). Axonin-1 expression in transfected areas of embryos treated with the EGFP plasmid alone (**H** and **I**) did not differ from untreated control embryos (not shown and **I**). The ratio of Axonin-1 staining was 3.4±2.2% (n = 5) for EGFP-control embryos and 2.3±0.9% (n = 4) in untreated control embryos. Bars: 50 µm in **A-D**, 200 µm in **E-H**.

expression (Figure 2.4B) in contrast to the homogenous appearance in a control embryo (Figure 2.4D). As expected, the vast majority of EGFP-expressing cells did not express Axonin-1 (Figure

2.4C). Consistent with the 50-fold excess of dsRNA molecules compared to the EGFP plasmid we found many cells that no longer expressed Axonin-1 but failed to express EGFP.

For the quantification of gene silencing we calculated the relative intensity of Axonin-1 staining in the electroporated area (Figure 2.4F) compared to the corresponding non-targeted area of the cerebellum (Figure 2.4G). A strong decrease of more than 60% in the Axonin-1 staining intensity was seen in the electroporated area of the cerebellum (identified by EGFP expression, Figure 2.4E) when embryos were injected and electroporated with axonin-1 dsRNA (Figure 2.4I). As expected there was no downregulation of Axonin-1 in the transfected area of embryos injected with the EGFP plasmid alone (Figure 2.4H).

In order to demonstrate the specificity of *ex ovo* RNAi, we monitored the expression of the two related cell adhesion molecules NgCAM and NrCAM that are both expressed in the developing chicken cerebellum (Figure 2.5). In agreement with published reports we found that Axonin-1 preceded NgCAM and NrCAM expression in postmitotic granule cells [37]. After downregulation of Axonin-1 no changes in the expression of NgCAM and NrCAM were observed (not shown). Taken together these results indicate that *ex ovo* RNAi with long dsRNA derived from axonin-1 effectively knocked-down Axonin-1 levels without affecting non-targeted but related genes expressed in the same area.

2.3.4 Downregulation of Axonin-1 by *ex ovo* RNAi resulted in aberrant extension of parallel fibers

For functional analysis of axonin-1 in the developing cerebellum, we injected the 678-bp-long dsRNA fragment that was used previously to analyze the function of axonin-1 in commissural axon guidance by *in ovo* RNAi [24]. Loss of Axonin-1 resulted in the failure of commissural axons to cross the midline [3,4]. Knocking down Axonin-1 in the developing cerebellum resulted in the failure of granule cell axons to extend parallel to each other and parallel to the pial surface (Figure 2.6). They were found to deviate from their normal trajectory, often even invading the outermost part of the EGL, where granule cell precursors proliferate (Figure 2.6C). In control embryos (Figure 2.6E) or on the contralateral side of experimental embryos (Figure 2.6D), Neurofilament staining was not found in the outer EGL. The failure of granule cell axons to extend in parallel manner with respect to each other and with respect to the pial surface was reflected in the irregular appearance of the molecular layer (Figure 2.6B). Its width was non-uniform and the fiber density seemed strongly reduced. Because we did not observe an increase of cell death compared to control embryos (Figure 2.3 and not shown) we took the apparent decrease in fiber number in the developing molecular layer as further evidence for the aberrant growth of parallel fibers.

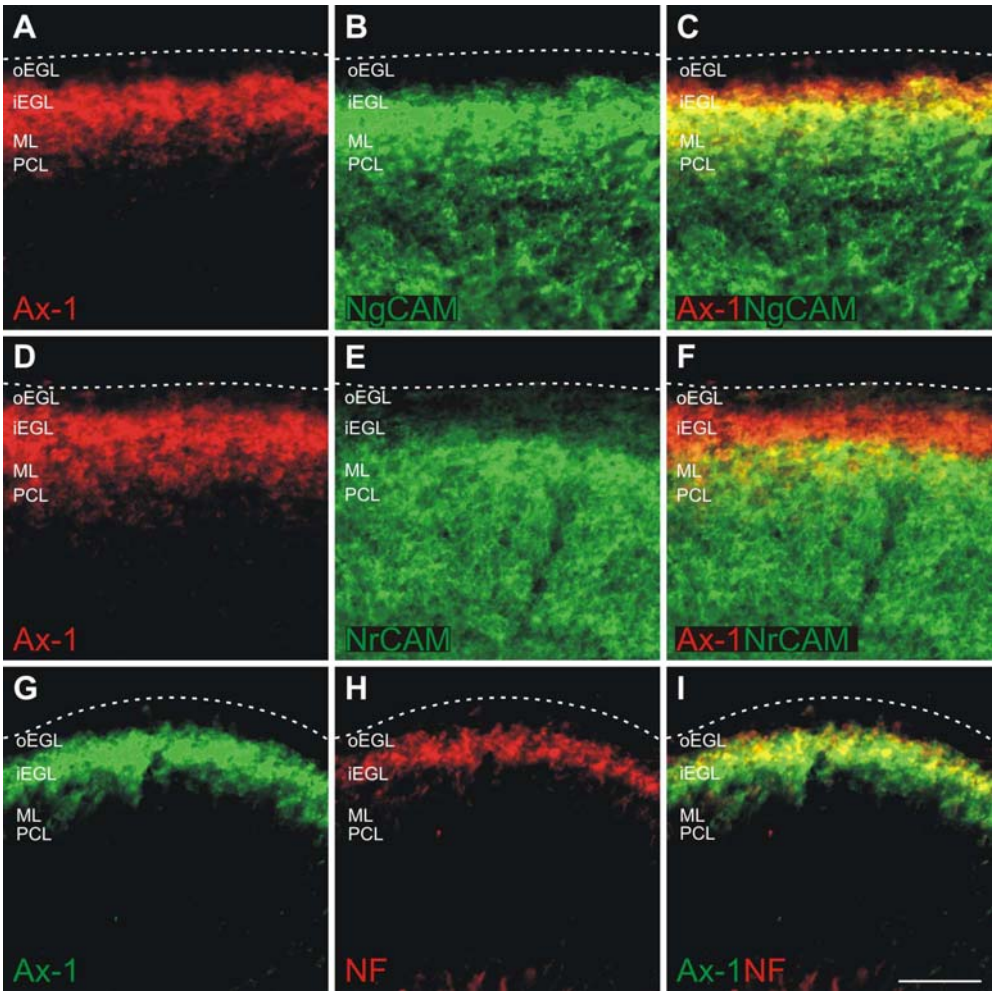

Figure 2.5 Axonin-1 expression in postmitotic granule cells precedes expression of NgCAM and NrCAM. Axonin-1 is a marker for postmitotic granule cells that start to extend processes in the inner EGL [6] (**A,D,G**). Double-staining of sagittal sections taken from HH35 brains demonstrated that Axonin-1 (**A**) did not co-localize with NgCAM (**B**; merged in **C**) in the inner EGL. There was co-localization on older parallel fibers in the ML, however. Double staining for Axonin-1 (**D**) and NrCAM (**E**) indicated that there was less overlap between Axonin-1 and NrCAM (**F**) compared to Axonin-1 and NgCAM (**C**). Axonin-1 reactivity (**G**) mostly overlapped with RMO270 reactivity (**H**) demonstrating that the vast majority of the Neurofilament signal at HH35 was generated by granule cells (**I**). Bar: 50 µm.

As it was impossible to count the number of aberrant fibers individually, we measured the intensity of Neurofilament staining in the developing molecular layer. As the width of the layer varies considerably depending on the antero-posterior position in the cerebellum, we measured the staining intensity as a ratio between the targeted and the corresponding contralateral (control) side. The comparison of Neurofilament staining intensity between the experimental and the control sides indicated a 38% decrease in axonin-1 dsRNA-treated embryos (n = 19 slices from 9 embryos) but no changes in EGFP-treated (n = 14 slices from 6 embryos) or untreated embryos (n = 10 slices from 4 embryos; Figure 2.6H).

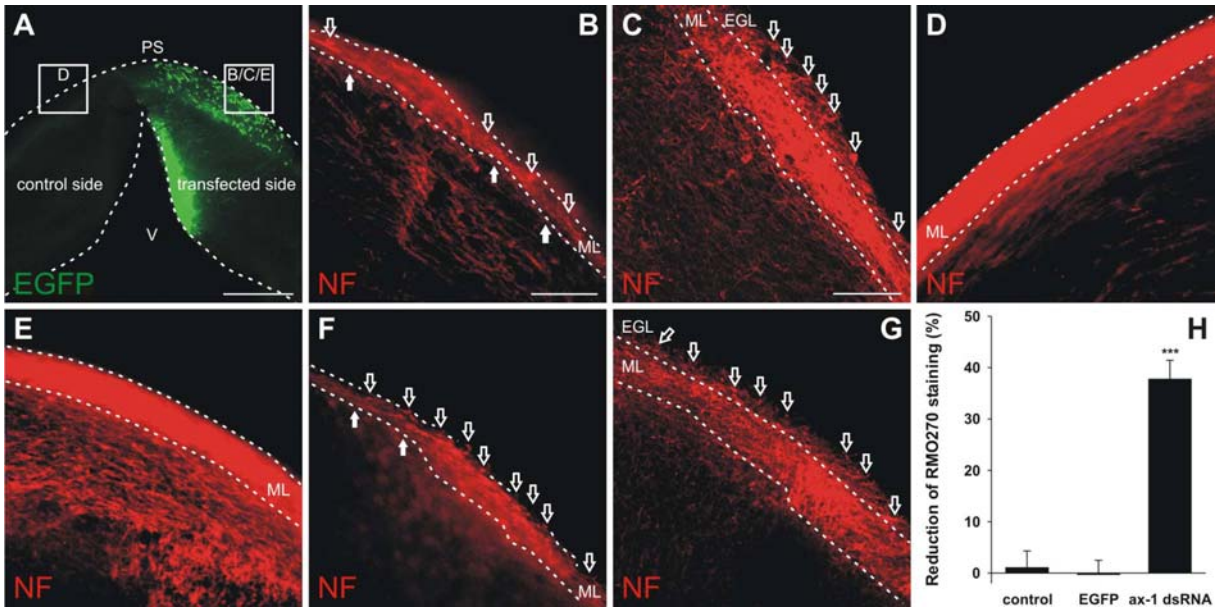

Figure 2.6 Silencing axonin-1 in the developing cerebellum induces aberrant trajectories of parallel fibers. Parallel fibers from areas outlined in (**A**) were analyzed in 250-µm-thick vibratome slices after downregulation of Axonin-1 (**A,B,C**). In the absence of Axonin-1 the formation of the molecular layer was severely disturbed in the transfected area (**B**). The width of the ML was irregular and fiber density was clearly reduced (white arrows in **B**). Most strikingly, axons of granule cells failed to extend in a parallel manner both with respect to each other and with respect to the pial surface (open arrows in **B**,**C**). A higher magnification of fibers leaving the developing molecular layer and projecting toward the pial surface is shown in (**C**). On the contralateral side of the cerebellum (**D**) the organization of the molecular layer and the density of the parallel fibers were indistinguishable from non-injected, age-matched control embryos (not shown). As expected, no difference between non-injected and EGFP-injected control embryos was observed (**E**). As an independent method for Axonin-1 perturbation we used antibodies to block Axonin-1 function at the protein level. The same phenotype that was obtained by *ex ovo* RNAi was reproduced with function-blocking antibodies (**F**,**G**). The effect of axonin-1 silencing by *ex ovo* RNAi was quantified (**H**). The relative fluorescence intensity after Neurofilament staining was determined as a measure for the decrease in both ML width and parallel fiber density. On average the staining intensity of the ML on the targeted side, i.e. in the absence of Axonin-1, was reduced by 37.8±3.6% compared to the non-affected control side (ax-1 dsRNA; n = 19 slices from 9 embryos). The staining intensities did not differ between the two halves of the cerebellum in embryos electroporated with the EGFP plasmid alone (EGFP; -0.3±2.8%; n = 14 slices from 6 embryos) or in non-treated control embryos (control; 1.1±3.2%; n = 10 slices from 4 embryos). P values were < 0.0001 for the comparison between both embryos treated with axonin-1 dsRNA and EGFP-expressing control embryos, and experimental embryos versus non-treated control embryos. EGL, external germinal layer; ML, molecular layer. Bars: 500 µm in **A**, 100 µm in **B**, **D**, **E** and **F**; 50 µm in **C** and **G**.

Measuring a decrease in Neurofilament staining underestimates the magnitude of the phenotype and is not informative with respect to the observed changes. Despite the fact that most Neurofilament staining is found in granule cells in the periphery of the cerebellum at this stage of its development (Figure 2.5H), a decrease in Neurofilament staining is not specific for granule cells. Therefore, we analyzed slices taken from experimental and control embryos with respect to the presence of

Neurofilament-positive structures in the pial layer of the cerebellum. In control embryos, both untreated and control-treated embryos no Neurofilament staining was found in the outermost layer of the developing cerebellum where granule cell precursors proliferate. In contrast, Neurofilament-positive fibers were observed in all slices that were taken from embryos injected and electroporated with axonin-1 dsRNA. These results were reproduced qualitatively in embryos treated with function-blocking anti-Axonin-1 antibodies (Figure 2.6F,G). Thus, perturbation of Axonin-1 function either by *ex ovo* RNAi or by injection of function-blocking antibodies resulted in aberrant growth of granule cell axons.

2.3.5 The aberrant growth of fibers is not caused by changes in granule cell development and maturation

To rule out an effect of aberrant granule cell development on parallel fiber formation we analyzed the migration of granule cells from the EGL through the developing ML. For this purpose we used Pax6 as a marker for granule cell somas [38] at HH35 (Figure 2.7A,B) and HH38 (Figure 2.7C,D). Downregulation of Axonin-1 did not interfere with granule cell migration (Figure 2.7B and D) compared to untreated controls (Figure 2.7A,C). Furthermore, the lack of Axonin-1 did not interfere with granule cell maturation. In particular, the number and position of proliferating granule cell precursors in the outer EGL was indistinguishable between control embryos (Figure 2.7E) and experimental embryos (Figure 2.7F). Thus, lack of Axonin-1 did not interfere with granule cell proliferation and migration.

2.3.6 Axonin-1 is required for parallel fiber formation but not for neurite extension

To address the mechanism underlying the observed failure of granule cell axons to extend in a parallel manner to each other and to the pial surface we investigated the role of Axonin-1 in neurite outgrowth promotion *in vitro*. When Laminin was used as a substrate granule cell axons grew almost exclusively on or in close contact with glia cells. Blocking Axonin-1 function did not interfere with this behavior nor change neurite length (Figure 2.8). Neurite length was 101.0 ± 7.3 µm for granule cells grown without antibodies added to the medium, 101 ± 9.7 µm in the presence of non-immune rabbit IgG, and 94.5 ± 7.6 µm in the presence of goat anti-Axonin-1 IgG.

These findings are supported by our *in vivo* results. Granule cells lacking Axonin-1 that were identified by the expression of EGFP still extended axons. However, the morphology and the direction of growth of these axons were severely perturbed (Figure 2.9). Most of the axons extended aberrantly toward the cerebellar surface and failed to form a T-shaped arbor.

Further confirmation for the absence of an effect of Axonin-1 on granule cell axon outgrowth was found when we coated culture dishes with purified Axonin-1. As described earlier [39,40], Axonin-1 supported axon outgrowth and the formation of characteristic large growth cones from dorsal root ganglion neurons. However, under the same conditions we did not detect neurite outgrowth from

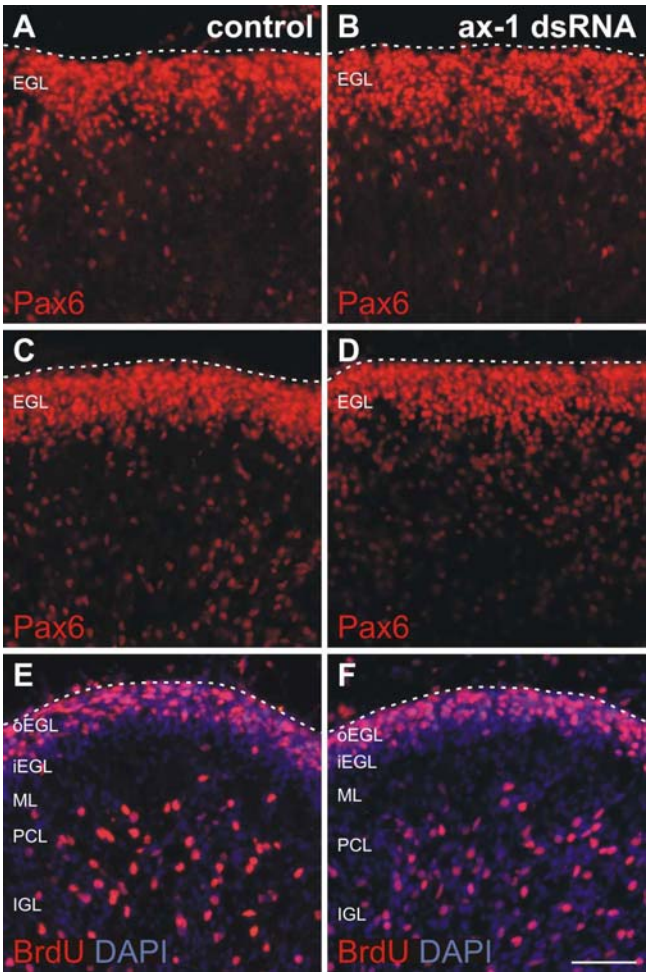

Figure 2.7 Downregulation of Axonin-1 does not interfere with granule cell development or migration. Perturbation of Axonin-1 expression did not interfere with granule cell migration from the EGL through the ML. No change in the distribution of Pax6-positive granule cells was detectable between untreated control embryos at HH35 (**A**) or HH38 (**C**) and embryos injected and electroporated with dsRNA derived from axonin-1 at HH35 (**B**) or HH38 (**D**). As expected, downregulation of Axonin-1 did not interfere with proliferation of granule cell precursors as Axonin-1 is only expressed after granule cells become postmitotic. Sagittal sections labeled with BrdU and counterstained with DAPI taken from control embryos at HH38 (**E**) and embryos lacking Axonin-1 (**F**) were not different. Bar: 50 µm.

granule cells (Figure 2.10). In agreement with our previous observations of granule cells in the presence of function-blocking antibodies (Figure 2.8), they extended neurites on Laminin, no matter whether they expressed Axonin-1 or not, i.e. when Axonin-1 was downregulated by *in vitro* electroporation before plating (Figure 2.10, G-L). In contrast, no axons were found on Axonin-1 used as a substrate (Figure 2.10, A-F). Therefore, we concluded that Axonin-1 was required for axon guidance but not for axon growth, in agreement with earlier observations made for commissural neurons from the dorsolateral spinal cord [41].

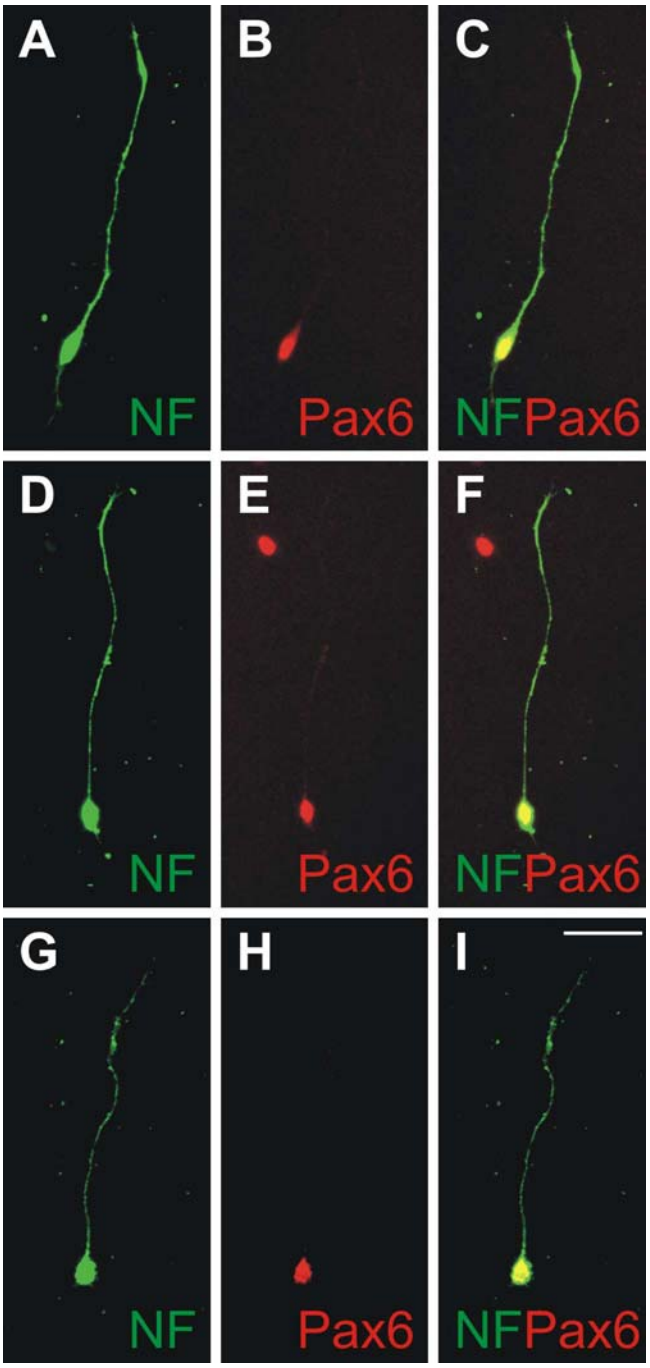

Figure 2.8 Axonin-1 is not required for neurite extension on Laminin. Granule cells were plated on Laminin in the absence of antibodies (**A-C**), in the presence of nonimmune antibodies (**D-F**), and in the presence of goat anti-Axonin-1 antibodies (**G-I**). Neurites were stained with a rabbit anti-Neurofilament antibody (**A,D,G**). Granule cells were identified by Pax6 (**B,E,H**). Merged images are shown in (**C,F,I**). Neurite morphologies and lengths did not differ under these conditions. Values were 101.0±7.3 µm (n = 27) in the absence of antibodies, 101.1±9.7 µm (n = 23) in the presence of non-immune, and 94.5±7.6 µm (n = 31) in the presence of anti-Axonin-1 antibodies. Bar: 25 µm.

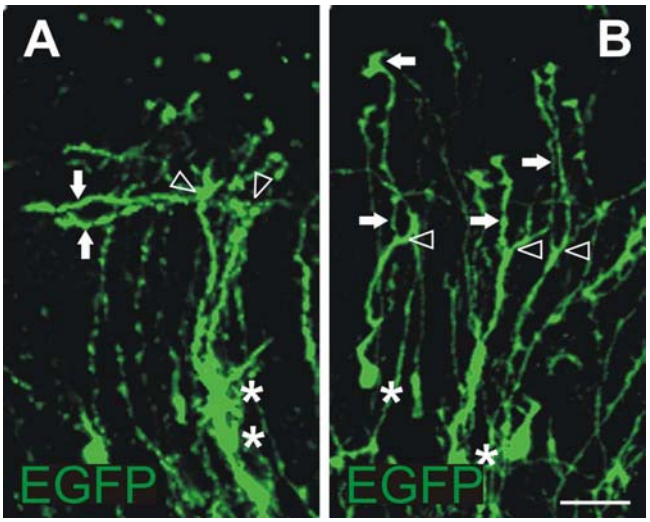

Figure 2.9 Guidance but not growth of granule cell axons is affected in the absence of Axonin-1 *in vivo.* Detailed analyses of coronal slices taken from control-injected embryos revealed correctly formed parallel fibers expressing EGFP (open arrows, **A**). In contrast, EGFP-expressing granule cells in embryos injected and electroporated with axonin-1 dsRNA and the EGFP plasmid did not extend their axons parallel to but rather in direction to the pial surface (arrows, **B**). Very few granule cells were found to develop a proper T-shaped axon in the absence of Axonin-1. Open asterisks label granule cells with correct T-shaped axons. White asterisks indicate granule cells that extend their axons in aberrant direction in the absence of Axonin-1. Open arrows indicate correctly extending parallel fibers, whereas white arrows label granule cell axons erroneously growing toward the pial surface. Bar: 25 µm.

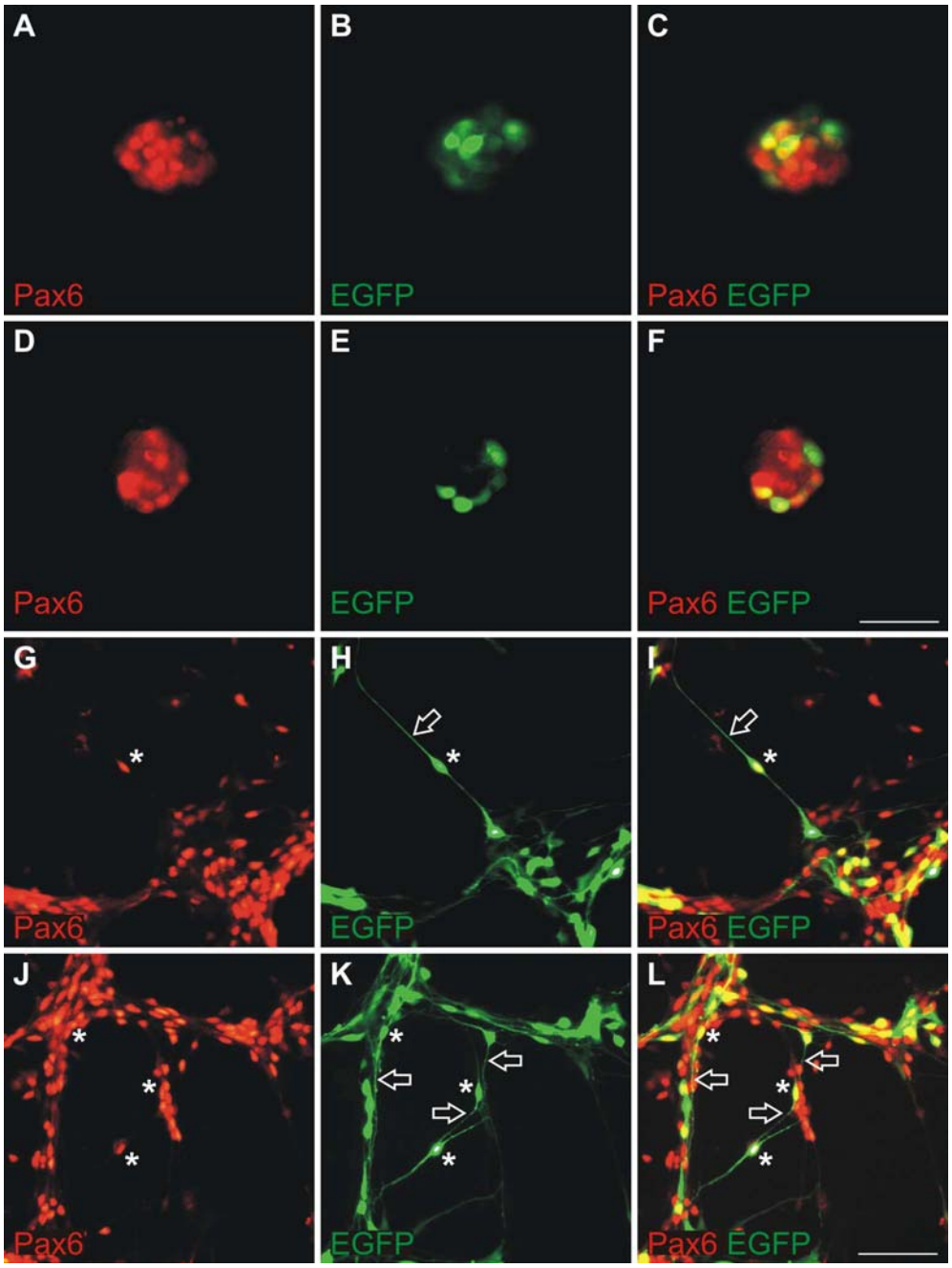

Figure 2.10 Axonin-1 does not promote outgrowth of granule cell axons. When granule cells were cultured on Axonin-1 as a substrate they failed to extend neurites (**A-F**), no matter whether they expressed Axonin-1 (**A-C**) or not (**D-F**). Granule cells were electroporated with dsRNA derived from axonin-1 and the EGFP plasmid *in vitro* before they were cultured on Axonin-1 substratum (**D-F**) and compared with cells electroporated with the EGFP plasmid only (**A-C**). In contrast, on Laminin both Axonin-1-expressing (**G-I**) and granule cells lacking Axonin-1 (**J-L**) extended axons. Granule cells were identified by their Pax6 expression (**A,D,G,J**). Electroporated cells expressed EGFP (**B,E,H,K**). Merged images are shown in (**C,F,I,L**). Asterisks indicated electroporated granule cells identified by EGFP and Pax6 expression. Open arrows label axons from Pax6-positive neurons that are also expressing EGFP. Bars: 25 µm in **A-F** and 50 µm in **G-L**.

2.4 Discussion

The chicken embryo was rediscovered as a modern model organism for developmental studies due to the use of electroporation for efficient gene transfer *in vivo* [32,34,42-44] (reviewed in [25,26]). With this method gain-of-function studies by spatially and temporally controlled expression of a target gene became possible [32,45-49]. However, loss-of-function phenotypes still depended on the availability of dominant-negative forms of candidate proteins. Therefore, the combination of *in ovo* electroporation and RNAi that allowed for temporally and spatially controlled gene silencing was an important step forward [24,30,50]. Functional studies by *in ovo* RNAi using long dsRNA, siRNAs, or shRNAs have been carried out in various parts of the CNS but also in other embryonic tissues [24,30,50-53] (reviewed in [25,26]). At later stages of development, the accessibility of the growing embryo through the small window in the eggshell is restricted and therefore the time window for *in ovo* electroporations closes at around E5.

In this study, we show that *ex ovo* electroporation and RNAi can be used for functional gene analysis at late developmental stages in the developing cerebellum. In the chick, the formation of the distinct cerebellar layers starts around HH34 (E8). The cerebellum represents an ideal system to investigate the multiple processes contributing to CNS development including cell proliferation [54], differentiation [55], migration [56,57], axon growth and guidance [58], as well as synaptogenesis [59].
Luo and Redies demonstrated that *ex ovo* electroporation can be used to transfect Purkinje cells [32]. Using a tungsten needle as cathode Purkinje cell progenitors were successfully electroporated after injection of a GFP plasmid into the ventricular system at E3.5. However, temporally and spatially controlled transfection of different cerebellar cell types was not possible at these early stages [32,34]. Furthermore, electroporation of antisense cDNA to induce a loss-of-function phenotype at E3.5 was not sufficient for functional readouts at later stages of cerebellar development (E11.5) [60]. Due to ongoing cell proliferation, transfected antisense cDNAs were diluted extensively, resulting in inefficient downregulation of the target gene. An additional problem with the early electroporation of antisense nucleotides, but also dsRNA for RNAi, may occur if a candidate gene exerts multiple functions at different stages of development. Early disruption of gene function would prevent any further functional analysis at later developmental stages. Therefore, our focus was to design appropriate conditions to extend the applicability of RNAi to older stages of embryonic development. Using this protocol we could efficiently downregulate Axonin-1 in granule cells of the EGL.

Despite the fact that some genes involved in cerebellar specification have been identified [12], the molecular basis of cerebellar development is still poorly understood [8,61]. A number of cell adhesion molecules of the immunoglobulin superfamily are expressed in granule cells, including NgCAM/L1 [37], NrCAM [37], Contactin/F3 [58,62], and Axonin-1/TAG-1 [6,7]. *In vitro* studies indicated an inhibitory role of Contactin/F3 in granule fiber outgrowth [63]. *In vivo*, parallel fibers extended in the absence of Contactin/F3 but their growth and contact with dendrites of Purkinje cells was aberrant [58]. Interestingly, only later extending parallel fibers were affected in contactin/F3 knockout mice,

growth of early extending fibers was normal, consistent with the late onset of contactin/F3 expression in granule cells compared to TAG-1/axonin-1 [6,64]. A forced early expression of Contactin/F3 under the TAG-1 promoter interfered with the correct formation of the molecular layer [64]. Reduced cerebellar size and defects in foliation were found in mice lacking both L1/NgCAM and NrCAM [37]. The defect was the result of a lack of granule cell survival rather than an effect on parallel fiber growth. Only very subtle cerebellar phenotypes were found in mice lacking only one of the two genes [37]. No cerebellar phenotype was reported in mice lacking TAG-1 [65].

Downregulation of Axonin-1 in the developing cerebellum specifically interfered with the formation of parallel fibers. This defect was not caused by inhibition of axon extension, as we found no effect of Axonin-1 on neurite growth *in vitro*. Furthermore, our analyses of cerebellar slices taken from embryos lacking Axonin-1 are consistent with an effect of Axonin-1 on guidance but not on growth of granule cell axons. Granule cells still extended axons after downregulation of Axonin-1, although their orientation was aberrant (Figure 2.9).

These results are consistent with earlier observations *in vitro* and *in vivo* made with commissural neurons from the dorsolateral spinal cord [41]. Axonin-1 was required for the choice of commissural axons between different substrates that supported axon extension but had no effect on axon growth *in vitro*. The same conclusion was drawn from *in vivo* studies where the absence of Axonin-1 resulted in the failure of commissural axons to cross the midline but did not affect neurite extension that was shown to depend on NgCAM and NrCAM. The effect of Axonin-1 on granule cell axon guidance is most likely due to a homophilic Axonin-1/Axonin-1 interaction [66,67], as granule cells do not express any of the known binding partners of Axonin-1, such as NgCAM (Figure 2.5B), NrCAM (Figure 2.5E), or Contactin/F3 [64] at the time when they start to extend their axons. A homophilic interaction of Axonin-1 is consistent with all our *in vitro* and *in vivo* results.

Conclusion

Axonin-1 expressed by granule cells as soon as they become postmitotic is required for the parallel arrangement of their axons but is not required for neurite extension. This is similar to the situation found for commissural axons of the spinal cord. They require Axonin-1 for pathfinding but not for elongation. In contrast, Axonin-1 was found to be required for guidance of sensory DRG axons *in vivo* [5] and to promote their outgrowth *in vitro* [39,40]. Taken together these results demonstrate that Axonin-1 acts in a context-dependent manner either as an axon guidance cue without affecting neurite outgrowth or as a molecule promoting neurite outgrowth. In the cerebellum, Axonin-1 expressed by postmitotic granule cells is required for the navigation of parallel fibers in the developing molecular layer.

2.5 Material and methods

Ex ovo culture for chicken embryos

Fertilized Hisex eggs were obtained from a local hatchery and pre-incubated for 2.5 days at 38.5°C with a humidity of at least 45%. After the egg had been positioned on the side for at least 20 minutes to allow for the embryo to float on top of the yolk, it was wiped with 70% ethanol and cracked on a sharp edge. The whole egg content was transferred carefully into a domed dish with a diameter of 80 mm and a depth of 40 mm. These dishes were produced for the food industry from oriented polystyrene (OPS; Bellaplast; Figure 2.1A). To minimize evaporation, the dish was covered with a lid. The *ex ovo* cultures were kept in an egg incubator at 38.5°C (Heraeus B12). To evaluate the survival rate at different stages of development we opened the incubator once a day to count survivors (Figure 2.1D). However, for routine experiments and best survival rate the incubator was not opened for the first 6 days of *ex ovo* culturing. Under these conditions the survival rate was much better (on average 62% at E8). Compared to the *ex ovo* culturing method described by Luo and Redies [34] the use of a domed dish was advantageous. We had better survival for a longer period of time in the domed polystyrene dishes. Contaminations were very rare (much less than 1%) although we did not work in a laminar flow hood.

Synthesis of dsRNA derived from axonin-1

For *in vitro* transcription 2 µg of the linearized (cut with BamHI and SacI; Roche) and purified plasmid encoding Axonin-1 (position 1620-2298 of the full-length cDNA; see [24] for details) were mixed with 0.8 µl 100 mM rNTPs (25 mM each; Roche), 0.5 µl RNasin (Promega), 2 µl Sp6 or T7 RNA polymerase (15 U/µl; Promega), and 2 µl 100 mM DTT in transcription buffer (final volume 20 µl). After 4 hours at 37°C, the DNA template was removed from the *in vitro* transcription mixture by digestion with 20 units RNase-free DNaseI (Roche) for 1 hour at 37°C. The synthesized ssRNA was purified by sequential extraction with acidic phenol-chloroform (25:24:1 vol/vol/vol phenol/chloroform/isoamyl alcohol) and chloroform/isoamyl alcohol (24:1). After precipitation with 100% ethanol, the ssRNA was washed with 70% ethanol, and dissolved in 20 µl PBS. To produce dsRNA, equal ng amounts of antisense and sense ssRNAs were mixed, heated for five minutes at 95°C, and then slowly cooled down to room temperature by switching off the heating block. For quality control, 1-µl samples were taken after each step and analyzed by gel electrophoresis. The dsRNA was stored at -80°C until further use.

Ex ovo injection and electroporation

Injections and electroporations were performed at E8 and E10. The embryos were staged according to Hamburger and Hamilton [35]. To have direct access to the embryo a small hole of 3 to 4 mm diameter was cut into the extraembryonic membranes above the eye. For positioning and stabilization of the head during injection and subsequent electroporation we used a hook prepared from a spatula (Figure 2.2A). Approximately 1 µl of the nucleic acid mixture consisting of a plasmid encoding EGFP under the control of the β-actin promoter (100 ng/µl), dsRNA derived from axonin-1 (500 ng/µl), and 0.04% (vol/vol) Trypan Blue (Invitrogen) dissolved in sterile PBS were injected into the cerebellum using a borosilicate glass capillary with a tip diameter of 5 µm (Figure 2.2A; World Precision Instruments). Depending on the depth of the injection different cerebellar layers could be targeted. To get transfection of all cerebellar layers the glass capillary was first inserted into the ventricular system and injection pressure was maintained during retraction. Before electroporation a few drops of sterile PBS were added to the embryo. For the electroporation a platelet electrode of 7 mm diameter (Tweezertrodes Model #520, BTX Instrument Division, Harvard Apparatus) was placed collaterally to the head of the embryo (Figure 2.2B). Six pulses of 40 V and 99 ms duration with one second interpulse intervals were applied using a square wave electroporator (ECM830, BTX).
For injections of function-blocking anti-axonin-1 antibodies, approximately 1 µl of the antibody solution (10 mg/ml with 0.04% Trypan Blue in PBS) was injected into the cerebellum three times every 12 hours.

Tissue preparation

One to four days after electroporation the embryos were sacrificed. The brain was removed and analyzed for EGFP expression using a fluorescence stereomicroscope (SZX12, Olympus). After fixation for two hours at room temperature in 4% paraformaldehyde in PBS, the brain tissue was rinsed in PBS and transferred to 25% sucrose in 0.1 M sodium phosphate buffer, pH 7.4, for cryoprotection. In this study, 30-µm-thick sagittal cryostat sections and 250-µm-thick coronal vibratome slices were used. For the preparation of cryostat sections, the brains were embedded with O.C.T Tissue-Tek in Peel-a-Way® disposable embedding molds (Polysciences), frozen in isopentane on dry ice and cut on a cryostat (Leica CM1850). The sections were collected on SuperFrost®Plus microscope slides. For the preparation of 250-µm-thick vibratome slices, the brain was embedded in 6.5% ultra low-melting agarose (Type IX, Sigma) and cut with an OTS-300-04 oscillating tissue slicer (Electron Microscopy Sciences). The slices were collected in PBS and the remaining agarose was removed before staining.

Immunohistochemistry

Immunostaining of cryostat sections was done essentially as described earlier [5]. We used the following primary antibodies: rabbit anti-GFP (1:250; Abcam), FITC-labeled goat anti-GFP 1:400; Rockland), anti-Calbindin (1:1000; Swant), both rabbit and goat anti-Axonin-1 (1:1000), goat anti-NgCAM, anti-NrCAM (both 1:1000), mouse anti-Neurofilament (RMO270; 1:1500; Zymed), rabbit anti-Neurofilament (1:250; Chemicon), and the monoclonal antibodies H5 recognizing Vimentin (supernatant) and Pax6 (2 µg/ml; both obtained from the Developmental Studies Hybridoma Bank). All antibodies were diluted in blocking buffer (10% fetal calf serum in PBS). Incubation was overnight at 4°C for primary and 90 minutes at room temperature for secondary antibodies. Secondary antibodies were: goat anti-rabbit-Alexa488 and donkey anti-goat-Alexa488 (1:250; Molecular Probes), goat anti-mouse-Cy3 (1:250), donkey anti-rabbit-Cy3 (1:200), or donkey anti-goat-Cy3 (1:250; all Jackson ImmunoResearch Labs). For staining of vibratome slices essentially the same protocol was used although incubation times were extended [68]. Slices were mounted in sterile PBS between two coverslips (24x24 mm) sealed with high vacuum grease (Dow Corning).

BrdU labeling

Embryos were injected with dsRNA derived from axonin-1 and the EGFP plasmid or with the EGFP plasmid alone and electroporated at HH34. After 4 days (HH38) 200 µl 50 mM BrdU in H_2O were pipetted onto the chorioallantois. After 3 h the embryos were sacrificed, the brains were dissected and prepared for cryostat sections as described above. For visualization of the incorporated BrdU, the sections were incubated in 50% form amide in 2xSSC for 1 to 2 h at 65 °C, rinsed twice in 2xSSC for 15 min followed by incubation in 2 N HCl for 30 min at 37 °C. Sections were rinsed in 0.1 M borate buffer (pH 8.5) for 10 min at room temperature, followed by PBS (six changes). BrdU was detected using the mouse anti-BrdU antibody from Sigma (1:200) using the protocol detailed above. Sections were counterstained with DAPI (5 µg/ml in PBS) for 20 min at room temperature.

TUNEL assay

Apoptosis was compared between *ex ovo* cultured control embryos, electroporated embryos injected with EGFP plasmid alone, or co-injected with dsRNA derived from axonin-1. As a positive control sections were treated with DNase I (300 U/ml; Roche) for 10 minutes at room temperature. To detect apoptotic cells, the ApoAlert DNA Fragmentation Assay Kit (Clontech) was used according to the manufacturer's instructions. The fragmented, fluorescein-labeled DNA was detected with an alkaline phosphatase-conjugated sheep anti-FITC antibody (1:1000; Roche) dissolved in 10% FCS/PBS. The alkaline phosphatase reaction was carried out as described earlier [69].

Quantification

Fluorescence intensities after Axonin-1 or Neurofilament staining were measured with the analySIS Five software from Soft Imaging System. Decrease of protein levels was calculated from the relative fluorescence intensities measured in the molecular layer of the transfected versus non-transfected areas. The staining intensity was then compared between embryos injected with dsRNA derived from axonin-1, embryos injected with the EGFP plasmid only, and untreated controls. For statistical analyses ANOVA with Bonferroni correction was used. Values are given as mean ± SEM. P values were < 0.0001.

***In vitro* assays**

Granule cells were collected by microdissection of the cerebellum at HH36. The EGL was separated from coronal slices by cutting through the ML. The tissue was collected in ice-cold HBSS (Hank's Balanced Salt Solution). After trypsinization and trituration cells were suspended in MEM containing 10% fetal calf serum, and Penicillin/Streptomycin and plated at a density of 50,000 cells per well of a 8-well LabTek slide. Slides were coated with 20 µg/ml Laminin or 50 µg/ml Axonin-1 essentially as described in [40] except that slides coated with Laminin were precoated with 100 µg/ml Poly-Lysine. After 48 h cells were fixed in 4% PFA for 1 h at room temperature. Cell cultures contained granule cells and Bergmann glia cells when coated on Laminin as determined by Pax6 and GFAP staining (not shown). On Axonin-1 substratum glia cells were not able to spread. Neurite lengths were measured and are representative for 4 different cultures. Antibodies were added to the culture medium at a concentration of 5 µg/ml.

***In vitro* electroporation**

Granule cells suspended in culture medium (see above), 40 ng/µl EGFP plasmid, and 5 ng/µl dsRNA derived from axonin-1 were electroporated using the BTX Safety Stand 630B and matching cuvettes connected to the BTX Electro Square Porator ECM830. After electroporation with 1 pulse of 5 ms duration at 112 V cells were kept at 37 °C for 5 min before plating. When cells were subject to electroporation before culturing they were plated at a density of 800,000 per well.

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3. Endoglycan plays a role in axon guidance and neural migration by downregulating cell-cell adhesion

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key words: sialomucin, commissural axons, Purkinje cells, ex ovo RNAi

(in preparation)

3.1 Summary

During development of the spinal cord dorsal commissural axons grow towards the floor plate, cross the midline, and then turn rostrally in a stereotypic manner. In a subtractive hybridization screen we have identified the transmembrane glycoprotein Endoglycan as a candidate guidance cue for postcommissural axons. Indeed, interference with Endoglycan function using in ovo RNAi causes perturbation of the floor-plate structure and pathfinding errors of commissural axons. Later in development, Endoglycan is expressed by migrating Purkinje cells in the cerebellum. Using ex ovo RNAi, we could demonstrate a requirement for Endoglycan in Purkinje cell migration in vivo. In the absence of Endoglycan Purkinje cells failed to migrate radially towards the pial surface. This in turn resulted in a significant decrease in cerebellar size and in a reduction of cerebellar foliation.

3.2 Introduction

Migration is an important aspect of neural development. Neurons are born in proliferative zones from where they migrate to their final destination. After their arrival, they send out axons that have to navigate through the tissue to find the target cells with which the neurons establish synaptic contacts. Thus, traveling does not stop it is just delegated to only a part of the cell. Intuitively it is clear that the same cues provided by the environment could be used for cells and axons to navigate through the tissue to find their target. However, we know relatively little about guidance cues for cells compared to guidance cues for axons. Both types of migration require cell-cell contacts. Not surprisingly, cell adhesion molecules were among the first axon guidance cues to be described (Rutishauser, 1985; Dodd and Jessell, 1988; Doherty and Walsh, 1989; Grenningloh and Goodman, 1992). Additional families of axon guidance cues were characterized in vertebrates and invertebrates, such as the Netrins (Baker et al., 2006; Cirulli and Yebra, 2007), the Semaphorins (Yazdani and Terman, 2006; Tran et al., 2007) with their receptors the Plexins (Negishi et al., 2005) and Neuropilins (Fujisawa, 2004), as well as the Ephs and ephrins (Egea and Klein, 2007). The most recent addition to the list of axon guidance cues were the morphogen families of the Wnts and Hedgehogs (Bovolenta, 2005; Stoeckli, 2006; Zou and Lyuksyutova, 2007).

One of the best studied models for axon guidance are the commissural neurons located in the dorso-lateral spinal cord. These neurons send out their axons toward the ventral midline under the influence of the long-range guidance cues BMP7 (Augsburger et al., 1999) and Netrin-1 (Kennedy et al., 1994; Serafini et al., 1996). BMP7, released by the roof plate, the dorsal midline of the spinal cord, repels commissural axons, whereas Netrin-1, released by the floor plate, the ventral midline, attracts commissural axons. The morphogen Sonic hedgehog (Shh) was shown to cooperate with Netrin in attracting commissural axons ventrally (Charron and Tessier-Lavigne, 2005). In addition to these so-called long-range guidance cues that define the overall direction of growth, short-range guidance cues that specify the actual pathway of growth are required. For dorsolateral commissural neurons Axonin-1 and NrCAM were shown to be attractive guidance cues (Stoeckli and Landmesser, 1995; Stoeckli et al., 1997; Fitzli et al., 2000; Pekarik et al., 2003). Slits and their receptors, the Robos, were shown to be required as negative signals involved in pushing axons out of the midline area (Long et al., 2004; Sabatier et al., 2004; Philipp et al., submitted). At least in mouse, members of the Semaphorin family are also involved in midline crossing as negative signals mediated by Neuropilin-2 (Zou et al., 2000). Once commissural axons exit the floor-plate area, they turn rostrally along the longitudinal axis of the spinal cord (Bovolenta and Dodd, 1990; Stoeckli and Landmesser, 1995). Guidance cues involved in this last step were discovered only recently both in mouse (Lyuksyutova et al., 2003) and chicken embryos (Bourikas et al., 2005). In the mouse, Wnt4 was shown to attract postcommissural axons rostrally, whereas Shh was identified as a repellent for postcommissural axons in the chick (Stoeckli, 2006; Zou and Lyuksyutova, 2007). The identification of Shh as a repellent for postcommissural axons was based on a subtractive hybridization screen for axon guidance cues influencing axonal navigation in the floor-plate area (Bourikas et al., 2005). In the same screen we identified another candidate that interfered with the rostral turn of postcommissural axons. This candidate gene turned out to be

Endoglycan. In the absence of Endoglycan commissural axons failed to turn rostrally upon floor-plate exit. Often they were observed to turn already inside the floor-plate area. Furthermore, the trajectory of commissural axons in the midline area was tortuous in embryos lacking Endoglycan but straight in control embryos.

Endoglycan is the most recently identified member of the CD34 family of sialomucins (Sassetti et al., 2000; Furness and McNagny, 2006). The family includes CD34, Podocalyxin (also known as PCLP-1, MEP21, or gp135), and Endoglycan (also known as Podocalyxin-like 2). They are single-pass transmembrane proteins with highly conserved transmembrane and cytoplasmic domains. A C-terminal PDZ recognition site is found in all three family members (Furness and McNagny, 2006). The hallmark of sialomucins is their bulky extracellular domain that is negatively charged due to extensive N- and O-glycosylation. Despite the fact that CD34 was identified more than 20 years ago, very little is known about its function. It has been widely used as a marker for hematopoietic stem cells and precursors. Similarly, Podocalyxin is expressed on hematopoietic stem and precursor cells. In contrast to CD34, Podocalyxin was found in podocytes of the developing kindney (Furness and McNagny, 2006). In the absence of Podocalyxin, podocytes do not differentiate resulting in kidney failure and thus perinatal lethality in mice (Doyonnas et al., 2001). Podocalyxin, but not CD34, is expressed in the developing and mature brain (Vitureira et al., 2005). Podocalyxin was shown to induce microvilli and regulate cell-cell adhesion via its binding to the NHERF (Na^+/H^+ exchanger regulatory factor) family of adaptor proteins that link Podocalyxin to the actin cytoskeleton (Nielsen et al., 2007). Like Podocalyxin, Endoglycan is expressed in the brain and in the kidney. Only low levels were found in hematopoietic tissues (Sassetti et al., 2000). Nothing is known about the function of Endoglycan.

The characterization of the temporal and spatial expression pattern of Endoglycan in the embryonic chicken cerebellum revealed its restriction to migrating Purkinje cells. We thus used a novel in vivo approach developed recently in our lab (Baeriswyl and Stoeckli, submitted) to study the role of Endoglycan in cerebellar development. The absence of Endoglycan resulted in the failure of Purkinje cells to migrate properly from the ventricular zone to their destination in the periphery of the cerebellum, where they normally form the typical Purkinje cell layer. This in turn resulted in a decrease in granule cell proliferation and in the stunted growth of the cerebellar folds.
Taken together, our results suggest a role for Endoglycan as a negative regulator of cell-cell adhesion in both commissural axon guidance at the ventral midline and Purkinje cell migration in the developing cerebellum.

3.3 Results

3.3.1 Endoglycan was identified as a candidate guidance cue for postcommissural axons

In a subtractive hybridization screen we identified differentially expressed floor-plate genes as candidate guidance cues directing axons from dorsolateral commissural neurons across the midline or along the longitudinal axis after their midline crossing. Candidates with an expression pattern that was compatible with a role in commissural axon navigation at the midline were selected for functional analysis using in ovo RNAi (Pekarik et al., 2003). One of these candidates that interfered with the correct rostral turning of commissural axons after midline crossing turned out to be Endoglycan. Endoglycan was expressed strongly by floor-plate cells at HH20 (Hamburger and Hamilton stage 20; Hamburger and Hamilton, 1951) but only weakly at HH26 (Figure 3.1). For functional analysis dsRNA was produced from the endoglycan cDNA fragment obtained in the screen and used for in ovo electroporation at HH18/19. The analysis of commissural axons' trajectories at HH26 by DiI tracing in „open-book" preparations (Figure 3.1C) revealed either failure to turn or erroneous caudal turns along the contralateral floor-plate border in embryos lacking Endoglycan (Figure 3.1E, F). Furthermore, axons were turning prematurely either before midline crossing or within the floor-plate area. To demonstrate specificity of Endoglycan downregulation and to verify that the phenotype was not due to an off-target effect, we used two additional cDNA fragments to produce dsRNA that were not overlapping with each other. All three fragments resulted in the same phenotypes. Pathfinding errors of postcommissural axons were seen at more than 80% of the injection sites in embryos lacking Endoglycan compared to 16% in control-treated and 5% in untreated control embryos (Figure 3.1H). There was no difference between embryos treated with dsRNA derived from the 3'UTR (Figure 3.1E and not shown) and those treated with dsRNA derived from the open reading frame of endoglycan (Figure 3.1F, G; 82.3±5.6% and 66±14.8%, respectively). Detailed analysis of the axonal morphology in the floor-plate area revealed a tortuous trajectory in embryos lacking Endoglycan (Figure 3.1G), whereas axons crossed the midline in a straight trajectory in control embryos (Figure 3.1D).

3.3.2 Endoglycan is the only member of the CD34 family of sialomucins that is expressed in the developing chicken nervous system

Endoglycan was first identified in a bioinformatics search for family members based on the homology of its transmembrane and cytoplasmic domains with Podocalyxin (Sassetti et al., 2000). In the mouse the sequence identity between Podocalyxin and Endoglycan in this region was found to be 44%. In the chicken the similarity between Podocalyxin (called Thrombomucin; McNagny et al., 1997) and Endoglycan is similar (40%; Figure 3.2A). There is no similarity between Endoglycan and CD34. In contrast, the comparison of the transmembrane and the cytoplasmic domains of human, mouse, and chicken Endoglycan reveals 89% identity between human and mouse and 78% between human and

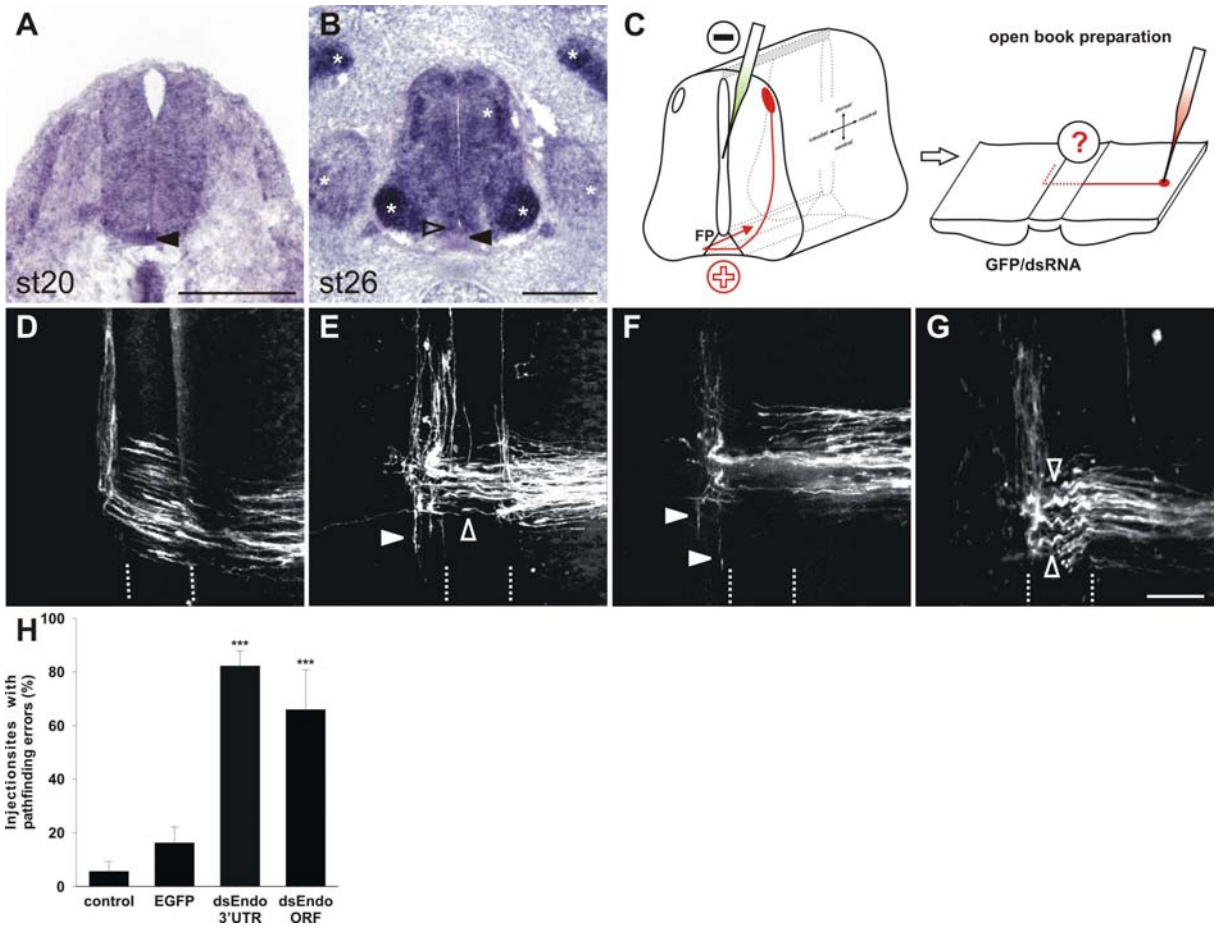


Figure 3.1 Endoglycan is required for the correct rostral turn of commissural axons after midline crossing. Endoglycan is expressed at high levels in the floor plate at HH20 (A) but only at low levels at HH26 (B). Commissural axon pathfinding was analyzed in "open-book" preparations (C). DsRNA derived from endoglycan together with a plasmid encoding EGFP, or the plasmid alone for controls, were injected into the central canal of the spinal cord at HH18/19. The position of the electrodes during electroporation is indicated. For analysis, the spinal cord was dissected from embryos at HH26, the roof plate was cut and the spinal cord was opened as indicated. Axonal trajectories were traced by DiI applied to the cell bodies of dorsolateral commissural neurons. In control embryos, commissural axons have crossed the floor plate and turned rostrally along the contralateral floor-plate border (D). In contrast, in embryos lacking endoglycan (E,F,G) commissural axons failed to turn along the contralateral floor-plate border or they turned randomly either rostrally or caudally (arrowheads in E and F). Occasionally axons were turning already inside the floor plate (open arrowhead in E). A closer look at the morphology of the axons in the floor plate revealed their tortuous trajectory across the midline (arrowheads in G).
The quantification of injection sites with pathfinding errors is shown in (H). Pathfinding errors were seen only at 5.6±3.7 % of the injection sites in untreated control embryos (n= 8 embryos). Control embryos injected and electroporated with the EGFP plasmid alone were found to show pathfinding errors at 16.2±6 % of the injection sites (n= 17 embryos). In contrast 82.3±5.6 % and 66±14.8 % of the injection sites in embryos injected with dsRNA derived from the 3'-UTR (n= 11 embryos) and the ORF of endoglycan (n= 6 embryos), respectively, showed aberrant pathfinding of commissural axons. P values < 0.001 for both groups compared to uninjected and EGFP-injected control groups. Bars: 200 µm in A, B; 100 µm in D, E, F, G.

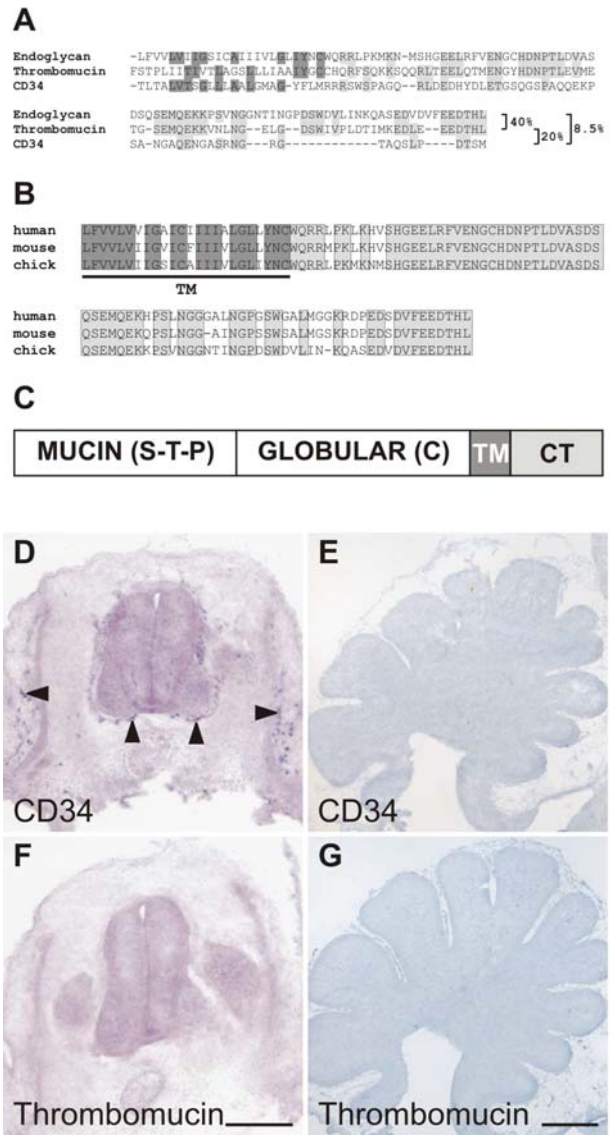


Figure 3.2 Endoglycan is the only CD34 family member that is expressed in the developing chicken nervous system. Endoglycan was identified as the third member of the CD34 family of sialomucins based on sequence similarity in the cytoplasmic domain with mouse podocalyxin (Sassetti et al., 2000). In the chick podocalyxin is called thrombomucin (McNagny et al., 1997). The similarity between chicken endoglycan and thrombomucin is 40% but only in the transmembrane and the cytoplasmic domain (A). No similarity is found between these domains when chicken endoglycan is compared to chicken CD34. In contrast, the sequence identity is very high when species orthologs are compared. Chick endoglycan is 78 % identical with human and 79 % identical with mouse, when only the transmembrane and the cytoplasmic domain are compared (B). The similarity in the extracellular domain is much smaller. The CD34 family of sialomucins do not share overall sequence identity but are grouped together due to their domain arrangement of an extracellular mucin domain followed by a globular domain, the transmembrane domain, and the cytoplasmic domain (C). The mucin domain is rich in serine, threonine, and proline. CT cytoplasmic tail, TM transmembrane domain. CD34 was not found to be expressed in the spinal cord (D) or in the cerebellum (E). A signal was seen in blood vessels surrounding the spinal cord and in the periphery, however (arrowheads). No expression was found for thrombomucin in spinal cord (F) or cerebellum (G). Bars: 200 µm in D, F; 500 µm in E, G.

chicken (Figure 3.2B). Thus, the conservation between species of the individual family members is very high at least in the transmembrane and cytoplasmic domain, but very low or not found when the family members are compared with each other. This is in contrast to the generally used definition of a protein family that is based on sequence similarity. The CD34 family members share the overall domain structure that consists of a mucin domain followed by a globular domain, the transmembrane, and the cytoplasmic domain (Figure 3.2C; Sassetti et al., 2000; Furness and McNagny, 2006).

There are no reports about the expression of CD34 in the nervous system (Furness and McNagny, 2006). In contrast, Podocalyxin was found in a subtractive hybridization screen for cDNAs enriched in upper cortical layers (García-Frigola et al., 2004). The analysis of its expression pattern revealed widespread expression of Podocalyxin in the developing and mature mouse brain (Vitureira et al., 2005).

We used in situ probes derived from ESTs of CD34 (Figure 3.2D, E) and thrombomucin (Figure 3.2F, G) to analyze their expression in the developing chicken nervous system. Neither CD34 nor thrombomucin were found in the spinal cord (Figure 3.2D, F), in the cerebellum (Figure 3.2E, G) or elsewhere in the brain (not shown). In contrast to CD34 and Podocalyxin/Thrombomucin, we found expression of Endoglycan in the developing chicken brain (Figure 3.3). High levels of expression were seen in the cerebrum, the cerebellum, and the spinal cord. Only low levels were found in non-neuronal tissues, such as muscle, heart, lung, and kidney. Thus, in chicken Endoglycan is the only sialomucin family member that is expressed in the developing nervous system.

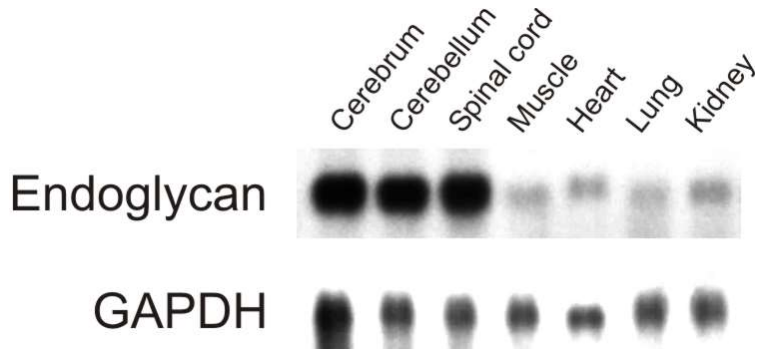


Figure 3.3 Endoglycan is expressed in the nervous system but only at low levels in non-neuronal tissues. Northern blot analysis of tissues taken from HH38 chicken embryos revealed its high expression levels in the cerebrum (brain without cerebellum), the cerebellum, and the spinal cord. Only low levels were found in muscle, heart, lung, and kidney. GAPDH was used as a loading marker. Endoglycan mRNA was detected at approximately 6.6kb and GAPDH mRNA at 1.3kb.

3.3.3 Lack of Endoglycan affects the morphology of the floor plate

Because the hallmark of sialomucins is their bulky, negatively charged extracellular domain with extensive glycosylation a role as regulators of cell-cell adhesion has been postulated. This together with our observation that commissural axons have a „corkscrew"-like phenotype in the midline area prompted us to analyze the morphology of the floor plate. Sections taken from control-treated and experimental embryos were taken from the lumbosacral level of the spinal cord at HH26 and stained for HNF3β/FoxA2 to label floor-plate cells, and for Axonin-1 to label commissural axons (Figure 3.4). In untreated (Figure 3.4A-C) and control-treated embryos (Figure 3.4D-F), HNF3β/FoxA2-positive cells were aligned to form the characteristic triangular shape of the floor plate. In particular, the ventral border of the floor plate, where commissural axons traverse the midline was smooth because all floor-plate cells were precisely aligned (Figure 3.4A, D). In contrast, floor-plate cells were no longer aligned to form a smooth ventral border in embryos lacking Endoglycan (Figure 3.4G, J). On the one hand, floor-plate cells were found to extend into the commissure formed by the Axonin-1-positive axons (Figure 3.4I and L). On the other hand, the floor plate appeared to have holes in embryos lacking Endoglycan. In addition, the floor-plate width was significantly narrower in embryos lacking Endoglycan in comparison to matched controls (Figure 3.4M). Taken together, these observations of an aberrant floor-plate morphology suggest a role of Endoglycan in the regulation of cell-cell contacts.

3.3.4 Endoglycan is expressed in migrating Purkinje cells and is required for their radial migration

At HH38, prominent expression of endoglycan was found in the cerebellum (Figure 3.5A,B), the tectum (Figure 3.5B), the forebrain (Figure 3.5A,B), the olfactory bulb (Figure 3.5A), and in the retina (Figure 3.5B). In the cerebellum (Figure 3.6), expression of endoglycan was first detected at HH36 in migrating Purkinje cells (Figure 3.6B). Expression was maintained during the formation of the Purkinje cell layer (Figure 3.6C-E) but was lost after cells had reached their final position at HH44 (Figure 3.6F).

Purkinje cells are born in the ventricular zone of the cerebellar anlage (Hatten and Heintz, 1995; Hatten, 1999). From there they migrate radially toward the periphery to form the distinct Purkinje cell layer (Figure 3.7A). In control embryos at HH38 the Purkinje cell layer is still more than one cell diameter in width but is clearly detectable in the periphery of the cerebellar lobes (Figure 3.7B,C). Very few Purkinje cells are found in the center of the lobes. The same is true in embryos injected with the EGFP-expression plasmid only (Figure 3.7D-F). In contrast, Purkinje cells were still found in the center of the lobes and close to the ventricular zone in HH38 embryos treated with dsRNA derived from endoglycan (Figure 3.7G-I). In addition the gross morphology of the cerebellum was severely compromised because individual lobes failed to separate. Overall the size of the cerebellum was significantly reduced (Figure 3.7K,L).

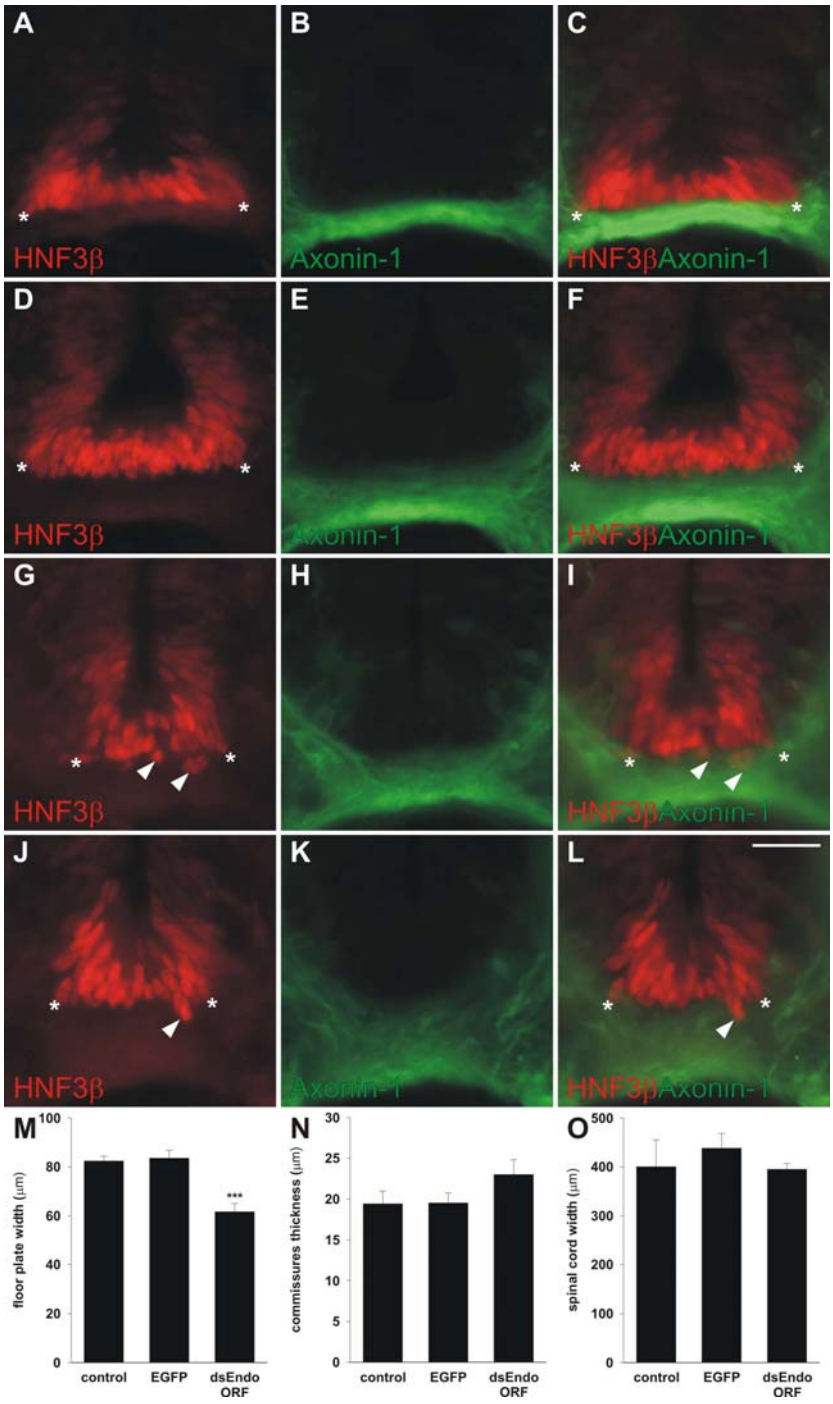


Figure 3.4 In the absence of Endoglycan the morphology of the floor plate is compromised. In untreated (A-C) and control-treated embryos (D-F) the floor plate is of triangular shape with floor-plate cells precisely aligned at the ventral border. There is no overlap between the floor plate (visualized by HNF3β staining; red) and the commissure (labeled by anti-Axonin-1 staining; green). The floor plate is no longer triangular in embryos lacking endoglycan (G-L). The floor-plate cells are not aligned ventrally (arrowheads in G, I, J, and L) resulting in holes in the floor plate. The width of the floor plate (indicated by asterisks) was measured (M) and compared with the width of the spinal cord. There was no significant difference in spinal cord width (O), but floor plates were significantly narrower in embryos lacking endoglycan (M; n= 7 embryos; $p<0.001$) compared to untreated (n= 6 embryos) and EGFP-injected control embryos (n= 6 embryos). The commissure had a tendency to be wider in experimental compared to control embryos but the effect was not statistically significant (N). Bar: 50 µm

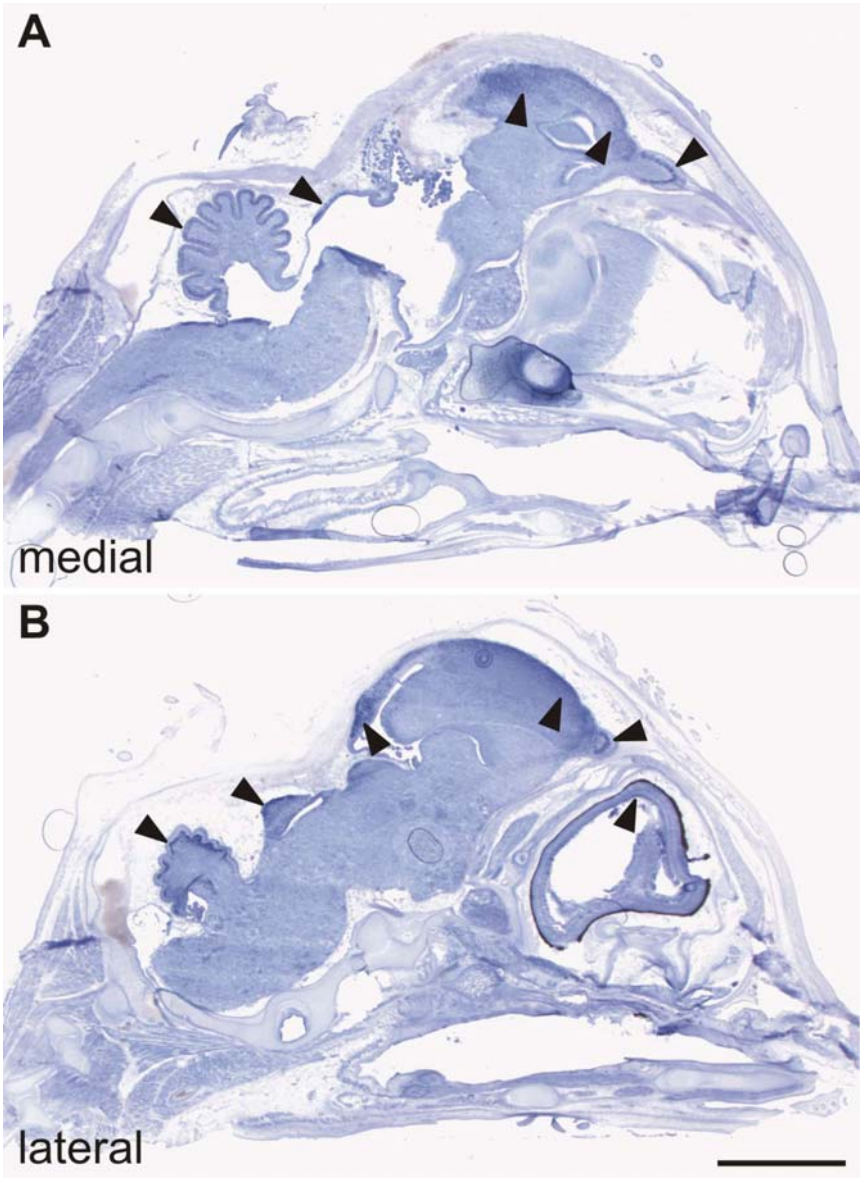


Figure 3.5 Endoglycan is expressed widely in the developing chicken nervous system. In situ hybridization revealed expression of endoglycan in the cerebellum (see Figure 3.6), in the tectum, in the forebrain, in the olfactory bulb, and in the retina. Bar: 2mm

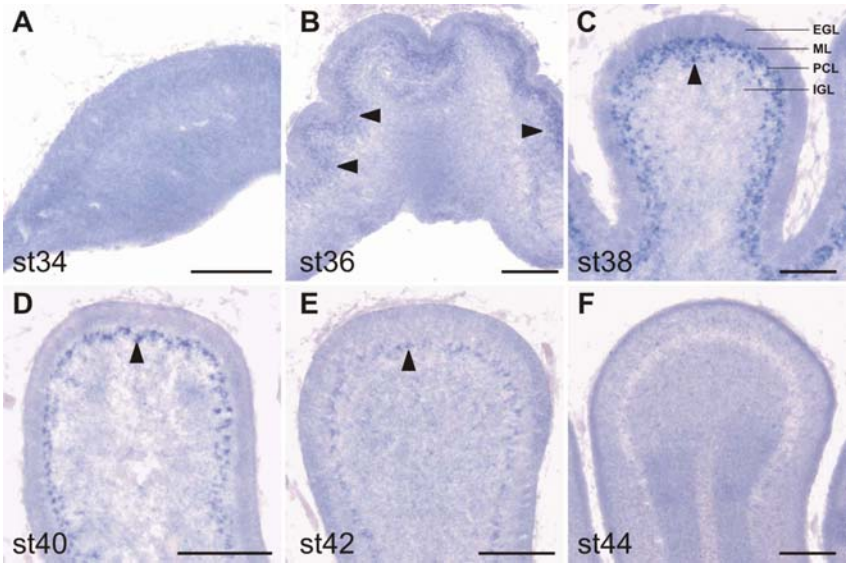
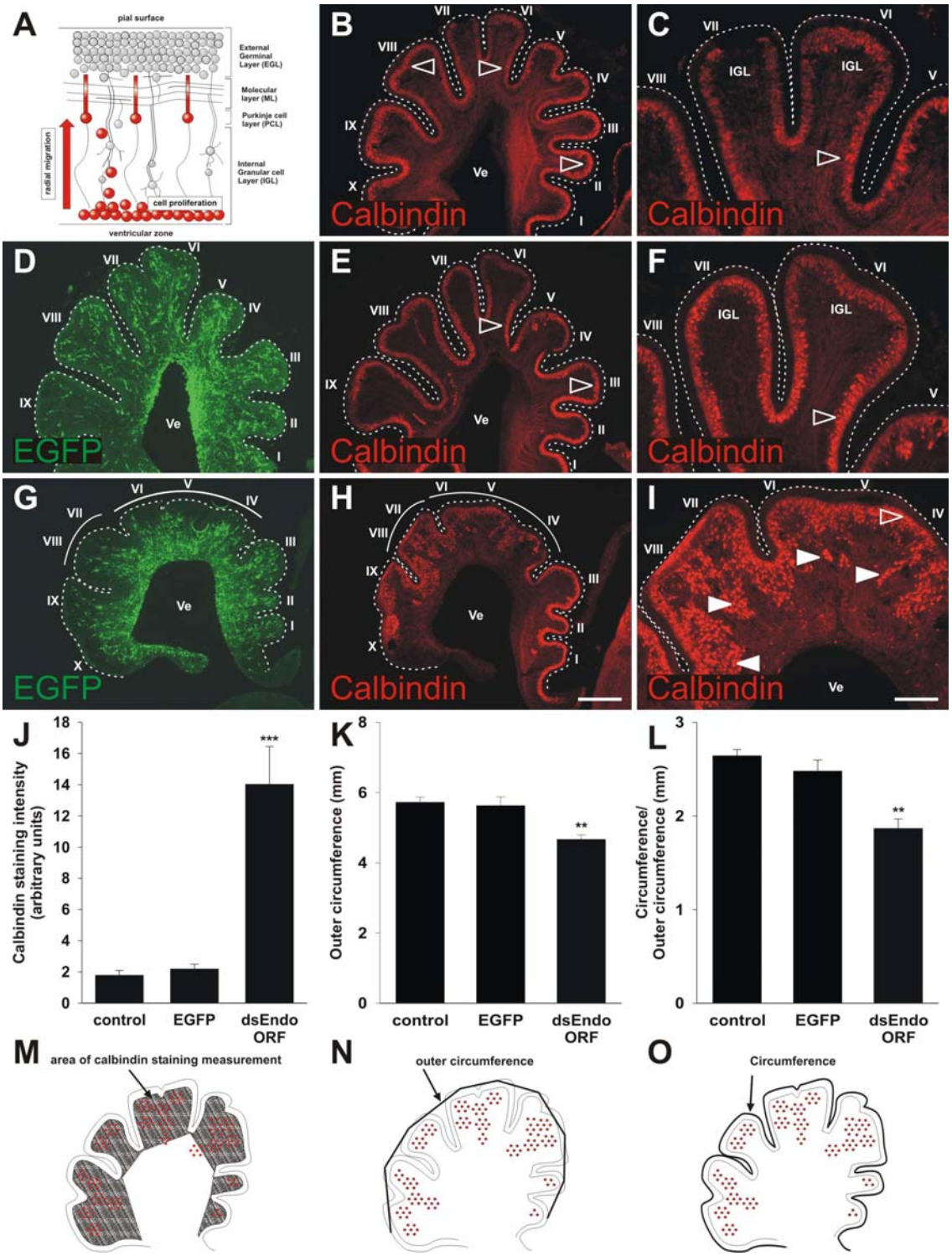


Figure 3.6 Endoglycan is restricted to Purkinje cells in the developing cerebellum. The temporal analysis of endoglycan expression in the developing cerebellum localizes it exclusively to migrating Purkinje cells. At HH34 (A), endoglycan is not yet expressed. At this stage, Purkinje cells are born in the ventricular zone. At HH36, Purkinje cells have migrated radially toward their final target layer in the periphery of the cerebelllum. They express endoglycan at this stage (arrowheads in B). At HH38, they have not yet formed the monolayer but express high levels of endoglycan (arrowhead in C). At HH40, the monolayer is almost achieved (D). Expression is still found at HH42, when the mature Purkinje cell monolayer is formed (arrowhead in E). After migration is completed and the monolayer is formed endoglycan is downregulated. No signal is left at HH44, one day before hatching (F). Bar: 100 µm in A-D, 200 µm in E, F.

Figure 3.7 Endoglycan is required for Purkinje cell migration. Purkinje cells are born in the ventricular zone of the cerebellum at HH34. They migrate radially toward the periphery of the cerebellar folds to form the Purkinje cell layer (A). In control embryos at HH38, the Purkinje cell layer visualized by Calbindin staining is clearly detectable although not fully matured to a monolayer (B and C). Control-injected embryos (D-F) were no different from untreated control embryos. EGFP was visualized with an antibody (D). Calbindin stains Purkinje cells in the periphery of the cerebellar folds (E and F). In the absence of Endoglycan (G-I) Purkinje cells fail to migrate and remain stuck in the center of the cerebellar folds (arrowheads in I). The failure of Purkinje cells to migrate radially and form the Purkinje cell layer was quantified by measuring fluorescence intensity for Calbindin in control (n= 5 embryos), EGFP-injected (n= 4 embryos), and dsRNA-treated embryos (J; n= 5 embryos). Calbindin staining intensity was measured as indicated in (M). The increase is highly significant for endoglycan dsRNA-treated embryos, $p < 0.001$. As a measure for the size of the cerebellum, the outer circumference was measured as indicated in (N). The experimental embryos had a smaller cerebellum compared to both control groups (K; $p<0.01$). In order to quantify the reduction in the number of folds that was obvious from the visual inspection of cerebellar sections, we measured the actual circumference of the cerebellum as indicated in (O) and divided it by the outer circumference. Control embryos had a ratio of 2.64±0.06 and 2.47±0.11, respectively. Embryos lacking endoglycan showed a clear reduction in the ratio between the two circumferences with a value of 1.8±0.1 (L), indicating that they had a lower number of cerebellar folds compared to control embryos, which have always 10 distinct folds. Bars: 500 µm in B, D, E, G, H; 200 µm in C, F, I.



3.3.5 Aberrant migration of Purkinje cells reduces granule cell proliferation

Purkinje cells are suggested to induce/regulate the proliferation of granule cells (Dahmane and Ruiz i Altaba, 1999; Wechsler-Reya and Scott, 1999; Wallace, 1999; Lewis et al., 2004). It was demonstrated that Sonic hedgehog released by the Purkinje cells affected proliferation of granule cells in the outer EGL (external granule cell layer). In turn reduced proliferation of granule cells was shown

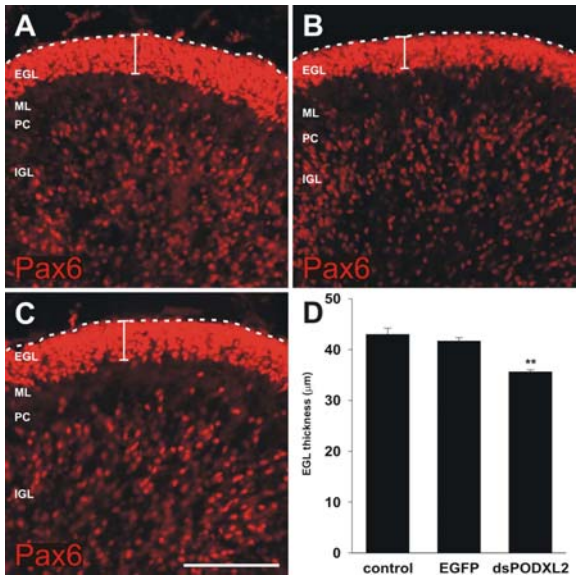


Figure 3.8 Embryos lacking Endoglycan have a thinner external granule cell layer. Pax6 is a marker for granule cells both in the EGL and in the IGL. A reduction in the width of the EGL was found for embryos treated with dsRNA derived from endoglycan (B, D; n= 3 embryos) compared to an age-matched untreated embryo (A; n= 4 embryos), when sections taken from the same relative position of the cerebellum were analyzed. No difference was found between untreated (A) and control-treated embryos, expressing EGFP (C; n= 3 embryos). $p<0.001$. Bar: 100 µm.

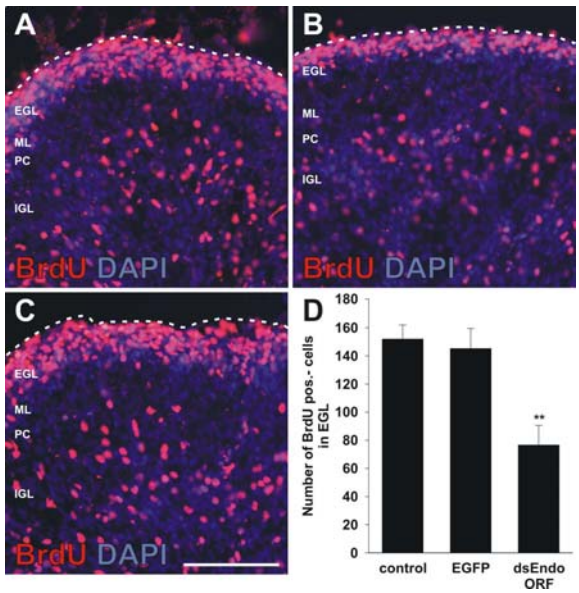


Figure 3.9 The reduction in EGL thickness is due to a reduction in granule cell proliferation. The proliferation of granule cells in the outer EGL was measured by BrdU incorporation. For this purpose, embryos were exposed to BrdU for 3 hours before they were sacrificed at HH38. The number of BrdU-positive cells in the outer EGL was compared between untreated (A; n= 6 embryos), EGFP-expressing control embryos (C; n= 4 embryos) and embryos lacking endoglycan (B; n= 5 embryos). The number of BrdU-positive cells was significantly reduced in embryos lacking endoglycan (D; $p<0.01$). Bar: 100 µm.

to result in changes in cerebellar morphology similar to the ones we observed after downregulation of Endoglycan (Figure 3.7). When we used Pax6 as a marker for granule cells, we found a thinner EGL in experimental embryos compared to control-treated and untreated embryos (Figure 3.8). This decrease in EGL width was due to a reduced proliferation rate of granule cells rather than apoptosis (Figure 3.9).

3.4 Discussion

We identified Endoglycan, a member of the CD34 family of sialomucins, in a screen for axon guidance cues involved in commissural axon pathfinding at the midline of the spinal cord. In the developing chicken cerebellum Endoglycan is expressed exclusively in Purkinje cells during their migration from the ventricular zone to their final destination between the molecular layer and the inner granule cell layer (Figure 3.6). In the absence of Endoglycan, Purkinje cells failed to migrate and accumulated in the center of the cerebellar folds (Figure 3.7). This observation suggests a role of Endoglycan as an anti-adhesive molecule that is supported by the structural features of sialomucins. The function of CD34 family members has not been characterized in detail but all the results obtained so far are compatible with an anti-adhesive role. One exception are reports from the lymph node cells, the so-called high endothelial venules (HEVs) where a very specific glycosylation patterns was implicated in the interaction of CD34 and Endoglycan with L-Selectin (Furness and McNagny, 2006). This function is unlikely to be important for the developing chicken nervous system, as the sequence containing the modified amino acids that are required for the interaction of Endoglycan with L-Selectin are not conserved in chicken. Therefore, in agreement with most published studies on the role of CD34 and Podocalyxin/Thrombomucin (for a review see Furness and McNagny, 2006) and in accordance with our observation we favor the model that suggests an anti-adhesive function of Endoglycan. The anti-adhesive effect is mediated by the negatively charged mucin domain. Similar to the role suggested for the polysialic acid modification of NCAM, the neural cell adhesion molecule (Brusés and Rutishauser, 2001), Endoglycan could lower cell-cell adhesion by increasing the distance between adjacent cell membranes due to repulsion caused by the bulky, negatively charged posttranslational modifications of its extracellular domains. A similar effect was found for PSA-NCAM in hindlimb innervation (Tang et al., 1994) and in the visual system, where retinal ganglion cell axons innervating the tectum were found to regulate axon-axon adhesion versus axon-target cell adhesion (Rutishauser et al., 1988). The same mechanism was found in motoneurons, where axon-axon versus axon-muscle fiber adhesion was a determining factor for the appropriate innervation pattern. In contrast to PSA-NCAM that continues to play a role in synaptic plasticity in the adult nervous system, the function of Endoglycan appears to be more restricted to development. Expression of Endoglycan ceased in the cerebellum after the mature wiring pattern was achieved.

At first sight the effect of Endoglycan on floor-plate morphology appears to suggest the opposite mechanism. Floor-plate cells are nicely aligned in control embryos but are protruding into the commissure in the absence of Endoglycan. Therefore, one might conclude that in the absence of Endoglycan cell-cell adhesion between floor-plate cells is compromised resulting in the observed structural changes. However, the observed changes can be explained differently by a mechanism that would again suggest an anti-adhesive role for Endoglycan. Commissural axons crossing the floor plate are suggested to do so by close interaction with short filopodial processes of floor-plate cells (Yaginuma et al., 1991). Because we could demonstrate that fasciculation is not required for midline crossing by commissural axons (Stoeckli and Landmesser, 1995) these interactions have to be plastic,

as later crossing populations must also be capable of interacting with the floor plate rather than just depending on fasciculation with pioneer fibers that crossed the floor plate earlier. This plasticity requires the downregulation of axon-floor plate adhesion. Thus, the function of Endoglycan in commissural axon guidance might be similar to its anti-adhesive role in Purkinje cell migration. In both cases the absence of Endoglycan would result in too much stickiness. In the cerebellum, too much stickiness prevents the Purkinje cells from migrating to their target layer. At the midline of the spinal cord, too much stickiness causes axons to adhere too much to floor-plate cells. This in turn could cause the observed phenotype because the failure to sever cell-axon contacts would result in floor-plate cells to be broken out of their regular arrangement (Figure 3.4). Consistent with this model, we observed a tortuous, corkscrew-like phenotype of axons in the floor-plate area that could reflect the excessive adhesion between floor-plate cells and axons (Figure 3.1G).

In summary, we propose a role for Endoglycan in axon guidance and neural migration as an anti-adhesive molecule that is fine tuning the balance between adhesion and de-adhesion. Precise regulation of adhesion is required in both processes and is fundamental for developmental processes that depend on a high degree of plasticity.

3.5 Material and methods

Cloning of an Endoglycan cDNA fragment (1028-1546bp)

A cDNA fragment from the coding sequence of Endoglycan (PODXL2) was obtained by RT-PCR using total RNA isolated from HH40 cerebellum. For reverse transcription 1 µg total RNA was mixed with 0.3 µl RNasin (Promega), 1 µl dNTPs (5 mM), 1 µl Random Nonamers, 1 µl DTT (Promega), 4 µl 5x Supercript II- buffer (Invitrogen) and adjusted with DEPC-treated water to a final volume of 20 µl. After incubation of 10 minutes at 70°C 1 µl Superscript II-RT (Invitrogen) was added and incubated for 1 hour at 42°C. The reaction was stopped by heating the mixture to 95°C for 10 minutes. Two µl of RT mixture were added to a PCR mixture consisting of 2.5 µl forward primer (10 µM; 5'-CAGACACGCAGACTCTTTC-3') and 2.5 µl reverse primer (10 µM; 5'-CTAAAGATGTGTGTCTTCCTCA-3'), 5 µl Expand Long Template Buffer 3 (Expand Long Template PCR System; Roche), 0.75 µl Expand Long Template Enzyme Mix (Expand Long Template PCR System; Roche), 2.5 µl dNTP-mixture (5 mM each), in a total volume of 50 µl. The PCR conditions were 35 cycles at 95°C for 30 sec, 57°C for 30 sec and 68°C for 3 min. The PCR product was cut with BamHI/BclI and cloned into pBluescript II KS (+/-). The ligation mixture consisting of 2 µl resuspended PCR product (400ng/µl), 0.2 µl vector solution (1000ng/µl), 1 µl Ligation mixture (QBiogene), 1 µl T4 Ligase (QBiogene) and ddH_2O to a final volume of 10 µl were incubated at 4°C overnight.

Preparation of DIG-labeled RNA probes and in situ hybridization

For *in vitro* transcription 1 µg of the linearized and purified plasmid for Endoglycan (EndoORF: 1028-1546pb, Endo3'UTR: 3150-3743bp and 5070-5754bp; numbers are derived from the human sequence), Thrombomucin (ChEST190L9) and CD34 (ChEST91D7) were mixed with 3 µl 10x DIG RNA Mix (Roche), 0.8 µl RNasin (Promega), 6 µl 5x Transcription Buffer (Promega), 3 µl T3 or T7 RNA polymerase (15 U/µl; Promega), 3 µl 100 mM DTT (Promega) and DEPC-treated ddH_2O to a final volume of 30 µl. After 4 hours at 37°C, the DNA template was removed from the *in vitro* transcription mixture by digestion with 20 units RNase-free DNaseI (Roche) for 1 hour at 37°C. The RNA transcript was precipitated with 3.8 µl 4 M LiCl and 113 µl absolute ethanol. After centrifugation at 12000 rpm and 4°C for 20 min, the pellet was washed with chilled 70% RNase-free ethanol and resuspeded in 50 µl DEPC-treated ddH_2O. For quality control, 1-µl samples were taken after each step and analyzed by gel electrophoresis. The DIG-labeled RNA probe was stored at -80°C until further use. For in situ hybridization the protocol published earlier was used (Mauti et al., 2006).

Northern Blot

Total RNA was extracted from cerebrum, cerebellum, spinal cord, muscle, heart, lung and kidney from HH38 embryos using the RNeasy Mini Kit (Qiagen). For separation 4.5 µg of total RNA per sample were loaded on a denaturing formaldehyde gel (1% agarose, 16% formaldehyde in 1x FRB consisting of 4.12 g MOPS, 0.65 g sodium acetate, and 2 ml 0.5M EDTA (pH8) diluted in 1 l DEPC-treated H_20). Before loading, the RNA samples were heated to 60°C for 10 minutes and subsequently chilled on ice for 2 minutes. The gel was run at 50V for 5 hours in FRB. The formaldehyde was washed out by rinsing the gel three times in DEPC-treated ddH_2O for 10 minutes. The RNA was blotted to a positively-charged nylon membrane (Roche) overnight using 10x SSC as a transfer medium. After rinsing the membrane twice with 2x SSC for 5 minutes, the RNA was crosslinked to the membrane using a UV-Stratalinker (Stratagene). To detect ribosomal RNA and the RNA ladder (Invitrogen) the membrane was incubated in 0.04% methylene blue supplied with 0.5 M sodium acetate for 10 minutes. After removing the methylene blue by rinsing three times in DEPC-treated ddH_2O for 10 minutes the RNA ladders as well as the ribosomal bands were marked with a pen. The membrane was incubated in 10 ml prehybridization buffer (50% formamide, 5x SSC, 2.5% blocking reagent, 0.1% SDS and 0.1% N-Lauroylsarcosine) for 2 hours at 68° using a hybridization oven (Thermo Electron). After removing the prehybridization buffer, 10 ml of hybridization buffer with 1.5 µg preheated DIG-labeled RNA probes for endoglycan and GAPDH were added and the membrane was incubated at 68°C overnight. The membrane was then washed twice with 2xSSC/0.1%SDS for 5 minutes at room temperature and twice with 0.1xSSC/0.5% SDS for 20 minutes at 68°C. For detection, buffer 2 (2% blocking reagent dissolved in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) was added for 2-3 hours at room temperature. After incubation with anti-digoxigenin-AP antibody dissolved in buffer 2 (1:10000; Roche) for 30 minutes at room temperature the membrane was washed twice in washing buffer (0.3% Tween 20 dissolved in 0.1 M maleic acid, 0.15 M NaCl, pH 7.5) for 20 minutes. Subsequently, detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH9.5) was applied for 2 minutes before adding CDP-star (1:100, 25 mM, Roche) for 5 minutes in the dark. For detection of the chemiluminescence a Kodak BioMAX XAR film was exposed for different durations.

***In ovo* RNAi**

For functional studies in the spinal cord, we silenced Endoglycan with three different long dsRNAs. They were produced from bp 1028-1546 of the ORF, bp 3141-3734 and bp 5061-5745 from the 3'UTR. The fact that we obtained the same phenotype with 3 different, non-overlapping dsRNAs derived from endoglycan confirms the specificity of the approach and the absence of off-target effects. DsRNA was produced as detailed in Pekarik et al., 2003. For description of the in ovo RNAi procedure, see chapter 16 in 'RNAi Design and Application' (chapter 5 of this PhD thesis; ed. Barik, S.; Methods in Molecular Biology, Humana Press, in press).

Ex ovo RNAi

To analyze the *in vivo* function of Endoglycan in the developing cerebellum *ex ovo* cultures of chicken embryos were prepared (Baeriswyl and Stoeckli, submitted). Injections and electroporations were performed at E8 (HH34). To have direct access to the embryo a small hole of 3 to 4 mm diameter was cut into the extraembryonic membranes above the eye. For positioning and stabilization of the head during injection and subsequent electroporation we used a hook prepared from a spatula. Approximately 1 µl of the nucleic acid mixture consisting of a plasmid encoding EGFP under the control of the β-actin promoter (100 ng/µl), dsRNA derived from the ORF of endoglycan (500 ng/µl), and 0.04% (vol/vol) Trypan Blue (Invitrogen) dissolved in sterile PBS were injected into the cerebellum using a borosilicate glass capillary with a tip diameter of 5 µm (World Precision Instruments). Before electroporation a few drops of sterile PBS were added to the embryo. For the electroporation a platelet electrode of 7 mm diameter (Tweezertrodes Model #520, BTX Instrument Division, Harvard Apparatus) was placed collaterally to the head of the embryo. Six pulses of 40 V and 99 ms duration were applied using a square wave electroporator (ECM830, BTX).

Tissue preparation and Analysis

To analyze commissural axon growth and guidance the embryos were sacrificed between HH25 and 26. The spinal cord was removed, opened at the roof plate ('open-book' preparation) and fixed in 4% PFA (Paraformaldehyde) for 1 hour at room temperature. To visualize the trajectories of commissural axons, Fast-DiI (5mg/ml, dissolved in methanol, Molecular Probes) was injected to the dorsal part of the spinal cord as described previously (Perrin and Stoeckli, 2000). The embryos were sacrificed for the analysis of the cerebellum one to four days after electroporation. The whole brain was removed and analyzed for EGFP expression using a fluorescence stereomicroscope (Olympus SZX12)**.** The brain tissue was fixed for two hours at room temperature in 4%PFA dissolved in PBS. After fixation, the brain tissue was rinsed in PBS and transferred to 25% sucrose in 0.1M sodium phosphate buffer, pH 7.4, for cryoprotection. In this study, 30 µm-thick sagittal cryostat sections and 250 µm-thick coronal vibratome slices were used for analysis. For the preparation of cryostat sections, the brains were embedded with O.C.T Tissue-Tek (Sakura) in Peel-a-Way® disposable embedding molds (Polysciences), frozen in isopentane on dry ice and cut on a cryocut (CM1850, Leica Microsystems, Heerbrugg, Switzerland). The sections were collected on SuperFrost®Plus microscope slides (Menzel-Glaeser).

Immunohistochemistry

Cryostat sections were rinsed in PBS at 37°C for 3 minutes followed by 3 minutes in cold water. Subsequently the sections were incubated in 20 mM lysine in 0.1 M sodium phosphate (pH 7.4) for 30

minutes at room temperature and rinsed in PBS three times for 10 minutes. The tissue was permeabilized with 0.1% Triton in PBS for 30 minutes at room temperature and then washed again three times with PBS for 10 minutes. To prevent unspecific binding of the antibody the tissue was blocked with 10% fetal calf serum (FCS) in PBS for one hour. Rabbit anti-GFP (1:250; Abcam), anti-axonin-1 (1:1000; Stoeckli et al., 1991), anti-Calbindin D-28K (CB38a; Swant) and mouse anti-HNF3β (supernatant; 4C7, DSHB) were dissolved in 10% FCS/PBS and incubated overnight at 4°C. After three washes in PBS, 10% FCS in PBS was applied again for one hour, followed by the incubation with goat anti-rabbit IgG-Alexa488 (1:250; Molecular Probes), donkey anti-rabbit IgG-Cy3 (1:200; Jackson ImmunoResearch) and goat ant-mouse IgG-Cy3 (1:250; Jackson ImmunoResearch) dissolved in 10% FCS in PBS for 90 minutes at room temperature. The tissue was rinsed 5 times in PBS for 12 minutes and then mounted in Celvol (Celanese). The staining of cryostat sections was analyzed with an upright microscope equipped with fluorescence optics (Olympus BX51).

BrdU labeling

Embryos were injected with dsRNA derived from endoglycan and the EGFP plasmid or with the EGFP plasmid alone and electroporated at HH34. After 4 days (HH38) 200 µl 50 mM BrdU in H_2O were pipetted onto the chorioallantois. After 3 h the embryos were sacrificed, the brains were dissected and prepared for cryostat sections as described above. For visualization of the incorporated BrdU, the sections were incubated in 50% formamide in 2xSSC for 1 to 2 h at 65 °C, rinsed twice in 2xSSC for 15 min followed by incubation in 2 N HCl for 30 min at 37 °C. Sections were rinsed in 0.1 M borate buffer (pH 8.5) for 10 min at room temperature, followed by PBS (six changes). BrdU was detected using the mouse anti-BrdU antibody from Sigma (1:200) using the protocol detailed above. Sections were counterstained with DAPI (5 µg/ml in PBS) for 20 min at room temperature.

Quantification

DiI injections sites with pathfinding errors were analyzed and counted using an upright microscope equipped with fluorescence optics (Olympus BX51). All measurements including floor-plate width, thickness of the commissure, spinal cord width, Calbindin fluorescence intensities, real and outer cerebellar circumference, EGL thickness, and number of BrdU positive cells were performed with the analySIS Five software from Soft Imaging System. All measurements were then compared between embryos injected with dsRNA derived from Endoglycan, embryos injected with the EGFP plasmid only, and untreated controls. For statistical analyses ANOVA with Bonferroni correction was used. Values are given as mean ± SEM. 1 asterisk: $P < 0.05$. 2 asterisks: $P < 0.01$. 3 asterisks: $P < 0.001$.

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4. Discussion and outlook

Potential use of *ex ovo* RNAi for cancer research

The chicken embryo represents an ideal model organism for developmental *in vivo* studies and has been used to reveal many basic principles of embryogenesis. However, due to lack of genetic tools the chicken embryo lost its importance. With the development of *in ovo* electroporation for efficient gene transfer in combination with RNAi functional gene analysis became possible and the increasing numbers of publications from the last few years underline the power of the chicken embryo as a model organism. One goal of my PhD thesis was to extend its versatility in particular for neurodevelopmental studies. Because the cerebellum represents an ideal system to study basic processes of brain development, ranging from cell proliferation to synapse formation, we decided to adapt gene transfer by electroporation and RNAi to the developing chicken cerebellum. We worked out a technique, *ex ovo* RNAi, for temporally and spatially controlled gene silencing *in vivo*. Using ex ovo RNAi, we analyzed the function of the cell adhesion Axonin-1/TAG-1 during cerebellar development. Although Axonin-1/TAG-1 has been identified more then twenty years ago (Stoeckli et al., 1989) and its function during CNS development has been studied extensively (Furley et al., 1990; Stoeckli and Landmesser, 1995; Stoeckli et al., 1997; Perrin et at., 2001), the role of Axonin-1/TAG-1 during cerebellar development remained unknown. *In vivo* data in combination with *in vitro* studies show that Axonin-1/TAG-1 expressed by postmitotic granule cells plays a crucial role for the navigation but not for the elongation of granule cell axons. Taken together, the combination of RNAi and the accessibility of the chicken embryo allow for both spatial and temporal control of gene silencing, and thus, represent a unique and important asset for functional gene analysis during embryogenesis.

Techniques for specific gene silencing developed and used for basic research contribute much to our understanding of disease development, and therefore, are important tools to assess future strategies for medical studies. Taking the advantages of *ex ovo* RNAi we plan to investigate Sonic hedgehog (Shh) signaling during cerebellar development *in vivo* and its potential role during formation of medulloblastoma. Medulloblastoma (MB) is the most common and malignant brain tumor in children and has been classified from the WHO as grade IV tumor due to its aggressive behavior (Gurney et al., 1996; McNeil et al., 2002). Originating from the cerebellum this highly aggressive tumor can invade other parts of the central nervous system (CNS) and peripheral nervous system (PNS) via cerebrospinal fluid, and therefore shows severe diagnostic symptoms, including headaches, morning vomiting, and ataxia (Fouladi et al., 1999; Polkinghorn and Tarbell, 2007). So far, therapies for MB such as surgical resection, chemotherapy or radiotherapy are limited in their specificity and suffer from low survival rates and severe long-term side effects including cognitive impairment, psychiatric disorders, endocrine dysfunction, and skeletal growth retardation (Feretti et al., 2005; Sakar et al., 2006, Polkinghorn and Tarbell, 2007). The present challenge in MB treatment is to improve specificity and to minimize side effects. Therefore, a better understanding of the molecular basis of MB development and the identification of potential targets is required to establish specific and efficient strategies for a MB therapy. Based on a number of mouse models, the regulation of granule cell development has been implicated in the formation of MB (Fogarty et al., 2005; Marino, 2005).

Although the cellular origin of MB is not yet clear, the majority of studies focus on the granule cells as a source of medulloblastomas. Besides the effect on granule cell proliferation of Sonic hedgehog that is secreted by Purkinje cells, Patched mutations that were found in MBs suggest that the Shh-Gli pathway and its components play a crucial role during MB development (Dahmane and Ruiz I Altaba, 1999; Wechsler-Reya and Scott, 1999, Zurawel et al., 2000). Despite extensive studies, the regulation of Shh signaling and additional mechanisms involved in MB development remains to be elucidated. *Ex ovo* RNAi will be a valuable tool to study the function of candidate genes for cerebellar development, and thus, may give rise to new targets for the development of therapeutic strategies to treat MB.

An outlook for Endoglycan function during nervous system development

For the first time, we describe a function for the glycoprotein Endoglycan during development of the central nervous system with focus on spinal cord and cerebellar development. Silencing of endoglycan by *in ovo* RNAi in the developing spinal cord resulted in aberrant commissural axon pathfinding at the floor plate, an intermediate target for dorsal commissural axons. We furthermore demonstrated that Endoglycan is important for the maintenance of the floor-plate morphology. However, the exact role of Endoglycan in floor-plate formation or maintenance has not been addressed in this study. Based on the literature, no conclusions about an underlying mechanism of Endoglycan function can be made due to the lack of functional data, except for its binding capacity for L-Selectin (Furness and McNagny, 2006, Fieger et al., 2003). However, binding to L-Selectin was dependent on a very specific glycosylation pattern of Endoglycan that was only found in high endothelial venules (Paavonen et al., 1992; Mitsuoka et al., 1997; Fieger et al., 2003). Furthermore, the sequence that was linked to this function of Endoglycan is not fully conserved in chicken. Podocalyxin (Thrombomucin in chicken) was shown to have an important role in the maintenance of filtration slits (podocyte foot processes) in the kidney glomerolus (Kerjaschki et al., 1984; Doyonnas et al., 2001). Although Podocalyxin is assumed to maintain this structure via negative-charge repulsion by the mucin-like ectodomain, experimental data for this model are lacking (Furness and McNagny, 2006). At the time when commissural axons cross the ventral midline, they are in close contact and wrapped by basal processes of floorplate cells (Yaginuma et al., 1991; Campbell and Peterson, 1993). Is the polarity of floor-plate cells compromised after interference with Endoglycan? To address this question it will be important to study the formation of tight and adherens junctions, e.g. by studying the distribution of tight junction-associated proteins. These studies will address the effect of floor plate cell polarity on axon guidance *in vivo*.

Furthermore, a direct effect of Endoglycan on commissural axon guidance cannot be excluded. It has been shown that CD34 regulates cell adhesion of bone marrow mast cells (Drew et al., 2005) most likely by blocking adhesion via steric hindrance with its the bulky, negatively charged mucin domain. Similarly, loss of Endoglycan function could lead to increased adhesion between commissural axons and floor-plate cells. Axons that form excessively strong interactions with floor-plate cells could disturb

the floor-plate morphology during midline crossing. Therefore, we will analyze the floor-plate morphology before commissural axons entered the floor plate in the absence of Endoglycan.

During cerebellar development Endoglycan is expressed by Purkinje cells during the time of their radial migration towards the pial surface. After perturbation of Endoglycan function Purkinje cells failed to migrate. As might be expected, cerebellar size and foliation were significantly reduced. A reduced number (*staggerer* mutant) or the erroneous location (reeler mutant) of Purkinje cells are known to have an effect on the granule cell population (Mallet et al., 1976; Mariani et al., 1977). Furthermore, the morphogen Shh secreted by Purkinje cells can regulate granule cell proliferation in the EGL (Dahmane and Ruiz I Altaba, 1999; Wallace, 1999; Wechsler-Reya and Scott, 1999). Therefore, we analyzed whether reduced cerebellar size and foliation in Endoglycan dsRNA-treated embryos is caused by reduced proliferation of granule cells, the largest neuronal population of the CNS. Indeed, we found a significant reduction in both thickness of the EGL and proliferation rate of granule cells in the EGL. The mechanism of Purkinje cell migration remains unclear. Do Purkinje cells stop to migrate or is the direction of Purkinje cell migration disturbed due to change in cell polarity in the absence of Endoglycan? Further studies will have to address the mechanism of Endoglycan function in cell polarity and cell migration.

5. Additional material and methods

Temporal Control of Gene Silencing by *in ovo* Electroporation

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Olivier Mauti and I developed the concept for this book chapter, wrote the text and acquired the images together. My contribution was the outline and final preparation of the figures.

Key Words: *in ovo* RNAi, *in ovo* electroporation, long dsRNA, chicken embryo, development, nervous system
Running Title: *In ovo* RNAi

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5.1 Abstract

The analysis of gene function during embryonic development asks for tight temporal control of gene expression. Classical genetic tools do not allow for this, as the absence of a gene during early stages of development will preclude its functional analysis during later stages. In contrast, RNAi technology provides the possibility to achieve temporal control of gene silencing especially when used with oviparous animal models. In contrast to mammals, reptiles and birds are easily accessible during embryonic development. We have developed approaches to use RNAi for the analysis of gene function during nervous system development in the chicken embryo. Although the protocol given here describes a method for gene silencing in the developing spinal cord, it can easily be adapted to other parts of the developing nervous system. The combination of the easy accessibility of the chicken embryo and RNAi provides a unique opportunity for temporal and spatial control of gene silencing during development.

5.2 Introduction

No matter whether you want to analyze the function of a number of candidate genes that you identified in a screen or whether you want to assess the function of your favourite gene, you may need an *in vivo* system that allows for a rapid detection of a possible phenotype during development. The analysis of gene function during development requires tight temporal control of gene silencing. Classic genetic tools will only allow for an assessment of gene function during the initial phase of gene activity. Additional activities during later developmental stages will not be within reach, as the lack of gene function during early stages will preclude the analysis of all subsequent stages. For this reason, specific gene silencing by RNAi (RNA interference) provides an exceptional tool for loss-of-function approaches during development in vertebrates. Up to now, different RNAi strategies have been established for mouse, rat, and chicken embryos (***1-4***, reviewed in ***5-7***). However, due to the limited accessibility of mouse and rat embryos during development, RNAi in combination with *in utero* electroporation is very difficult and requires special equipment and expert training. Therefore, the use of mammals as a model organism for developmental studies is limited. In contrast to mammals, the chicken is easily accessible for *in vivo* manipulations during embryonic development. With the establishment of *in ovo* electroporation, as an efficient method for nucleic acid transfer, and *in ovo* RNAi, as a method for gene silencing, the chicken embryo has been turned into a unique model organism for the efficient functional characterization of genes involved in developmental processes (***4, 8-12***, reviewed ***6***). *In ovo* RNAi using long dsRNA, shRNA, or siRNA has been used for a variety of functional studies in different parts of the CNS but also in other embryonic tissues (***4, 13-17***, reviewed in ***5***).

Here, we provide a detailed protocol for the silencing of a candidate gene during early development of the spinal cord by *in ovo* RNAi. A particular advantage of *in ovo* RNAi is the fact that long dsRNA can be used for the induction of loss-of-function phenotypes. Unlike adult tissue or cell lines, embryos do not respond to long dsRNA with unspecific inhibition of protein synthesis and apoptosis (***18, 19***). Therefore, there is no need for lengthy selection of an efficient siRNA or shRNA. Any cDNA fragment or expressed sequence tag (EST) can be used to produce dsRNA by *in vitro* transcription. Because the chicken genome was fully sequenced in 2004, it can directly be compared with the human, mouse, or rat genome and the identification of orthologs has become very easy (***20***). Therefore, *in ovo* RNAi offers the possibility to study candidate genes identified in other species using commercially available Chicken ESTs for the synthesis of the dsRNA.

In the protocol reported here, long dsRNA is injected into the central canal of the developing spinal cord (**Figure 5.1A**). Subsequently, the embryo is exposed to an electric field for efficient transfection of selected cell populations (**Figure 5.1B**). Depending on the time point and the position of the electrodes different neuronal populations within the spinal cord (**Figure 5.1C** and **1D**) but also of the peripheral nervous system can be targeted. Furthermore, this method allows for knockdown of several genes by injecting a mixture of different dsRNAs. Thus, *in ovo* RNAi represents an efficient and inexpensive method to alter the expression of specific genes in a temporally and spatially controlled manner.

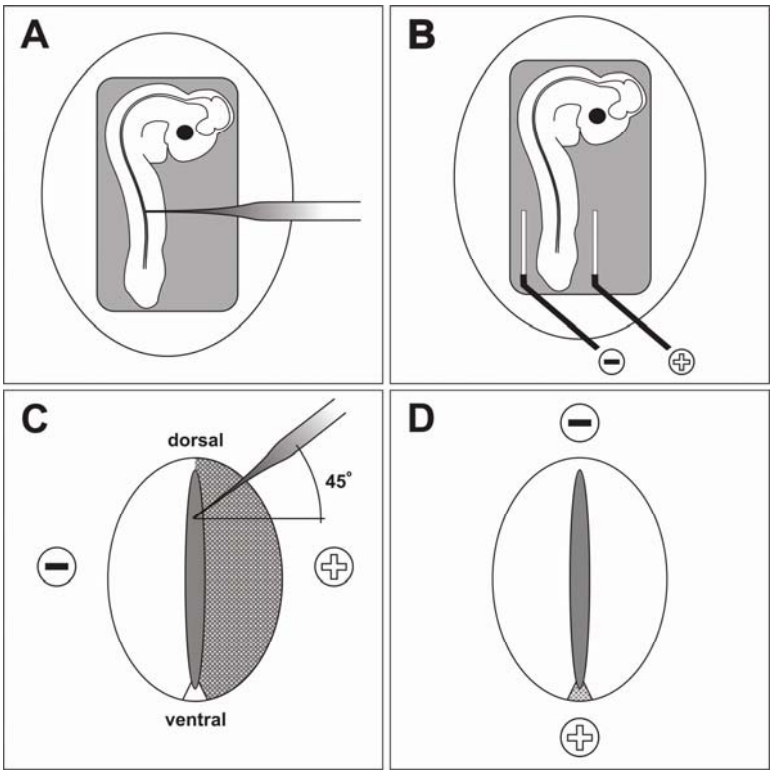

Figure 5.1 *In ovo* electroporation. The chicken embryo is made directly accessible through a window in the egg shell for the injection of nucleic acids into the central canal of the spinal cord (A). The electroporation permeabilizes the cell membrane and therefore allows for the efficient uptake of RNA or DNA (B). Depending on the position of the electrodes with respect to the embryonic body axis, different tissues can be targeted: A parallel position of the electrode to the spinal cord results in a unilateral transfection (checkered area in C). Within the applied electric field, the injected nucleic acids migrate towards the anode due to the negative charge of RNA and DNA. Therefore, the untransfected side of the spinal cord (left side in C) can be used as an internal control. The capillary should be kept at a 45° angle for injection. Placing the electrodes over the dorsal (cathode) and the ventral (anode) midline of the spinal cord results in efficient targeting of floor-plate cells (checkered area in D).

5.3 Materials

5.3.1. Preparation of dsRNA by in vitro Transcription

1. Heating block at 95°C.
2. Equipment for gel electrophoresis.
3. *Bam*HI restriction endonuclease (10 U/µL Roche, Basel, Switzerland).
4. *Sac*I restriction endonuclease (10 U/µL Roche, Basel, Switzerland).
5. RNasin (40 U/µL; Promega, Madison WI, USA).
6. SP6 and T7 RNA polymerases (15 U/µL; Promega, Madison WI, USA).
7. RNase-free DNaseI (10 U/µL; Roche, Basel, Switzerland).
8. 5X Transcription buffer (Promega, Madison WI, USA).
9. 100 m*M* rNTPs (25 mM each rNTP; Roche, Basel, Switzerland).
10. 100 m*M* DTT (Promega, Madison WI, USA).
11. 0.5 *M* EDTA (pH 8.0).
12. 7.5 *M* ammonium acetate.
13. Phenol:chloroform:isoamyl alcohol (25:24:1 vol/vol/vol; pH 7.6-8.0).
14. Acidic phenol:chloroform:isoamyl alcohol (25:24:1 vol/vol/vol; pH 4.0).
15. Chloroform:isoamyl alcohol (24:1 vol/vol).
16. 100% ethanol.
17. DEPC-treated double-distilled water (ddH_2O) (1:1000 vol/vol).
18. 70% ethanol in DEPC-treated ddH_2O.
19. Phosphate-buffered saline (PBS, DEPC-treated, 1:1000 vol/vol): 137 m*M* NaCl, 2.7 m*M* KCl (*see* **Note 1**), 8 m*M* Na_2HPO_4, 1.5 m*M* NaH_2PO_4 (pH 7.4).
20. RNaseZAP (Sigma-Aldrich, St. Louis MO, USA).

5.3.2. Windowing the Eggs

1. Fertilized Hisex eggs were obtained from a local hatchery.
2. Preincubator: 38-39°C, 45% humidity (JUPPITER 576 SETTER+HATCHER, F.I.E.M., Guanzate, Italy; *see* **Note 2**).
3. Incubator: 38-39°C, 45% humidity (Heraeus/Kendro Model B12, Kendro Laboratory Products, Hanau, Germany; *see* **Note 2**).
4. Egg-Lume Candler (Brinsea Products Ltd, Sandford, England).
5. Heating plate, 80°C, to melt paraffin.
6. Soldering iron.
7. Scalpel for drilling holes.
8. 10 mL syringe with needle (Sterican 100, Ø 18G, B. Braun Melsungen AG, Melsungen, Germany).
9. Small scissors for cutting the egg shell (Fine Science Tools Inc., Foster City, CA, USA).
10. Paraffin (Paraplast Tissue Embedding Medium, Oxford Labware, St. Louis, MO, USA).

11. Coverslips, 24 x 24 mm (VWR International AG, Dietikon, Switzerland).
12. Kleenex.
13. Scotch tape.
14. 70% ethanol.

5.3.3. In ovo Injection and Electroporation

1. Spring scissors and forceps (Fine Science Tools Inc., Foster City, CA, USA).
2. Electroporator (Electro Square Porator ECM830, BTX Instrument Division, Harvard Apparatus Inc., Holliston MA, USA; *see* **Note 3**).
3. Platinum electrodes (4 mm length, 4 mm between anode and cathode, BTX Instrument Division, Harvard Apparatus Inc., Holliston MA, USA; *see* **Note 3**).
4. Needle puller (PC-10, Narishige Co., LTD., Tokyo, Japan).
5. Borosilicate glass capillaries (outer Ø / inner Ø: 1.2 mm / 0.68 mm; World Precision Instruments, Sarasota FL, USA).
6. Polyethylene tubing (Ø 1.24 mm).
7. Filter 0.2 µm (Sarstedt, Sevelen, Switzerland).
8. Reporter plasmid: EGFP under the control of a chicken β-actin promoter.
9. Trypan Blue solution 0.4% (Invitrogen, Carlsbad CA, USA).

5.4 Methods

5.4.1. Synthesis of Long dsRNA

A cDNA fragment of a candidate gene cloned into a standard plasmid containing SP6 (or T3) and T7 promoters flanking the insert can be used for the synthesis of long dsRNA by *in vitro* transcription. Here, we synthesized dsRNA from a 678 bp cDNA fragment (1620-2298 bp) encoding Axonin-1 cloned in the pSP72 vector using SP6 and T7 promoters flanking the insert (*see* **Note 4**).

1. Linearize 10 µg of the plasmid with 20 U BamHI and SacI restriction endonuclease, respectively, for 1 hour at 37°C.
2. For *in vitro* transcription, mix 2 µg of the linearized plasmid with 0.8 µL 100 m*M* rNTPs, 0.5 µL RNasin, 2 µL SP6 or T7 RNA polymerase, 4 µL 5x Transcription buffer, 2 µL 100 m*M* DTT and add DEPC-treated ddH_2O to a total volume of 20 µL (*see* **Note 5**).
3. Incubate the *in vitro* transcription mixture for 2 hours at 37°C (T7 RNA polymerase) and 40°C (SP6 RNA polymerase), respectively (*see* **Note 6**).
4. Remove the DNA template from the *in vitro* transcription mixture by adding 2 µL RNase-free DNaseI and incubation at 37°C for 1 hour (*see* **Note 6**).
5. Add 20 µL of DEPC-treated ddH_2O and mix well.
6. Add a mixture of 2 µL 0.5 M EDTA and 22 µL 7.5 M ammonium acetate. Mix well.
7. Purify the synthesized ssRNA with 1 volume of acidic phenol:chloroform:isoamyl alcohol and subsequent extraction with 1 volume of chloroform:isoamyl alcohol.
8. Precipitate with 2.5 volumes 100% ethanol for at least 1 hour at –80°C.
9. Centrifuge for 30 min at 4°C and 20,000 x g.
10. Wash the RNA pellet with 70% ethanol and spin down.
11. Air dry the pellet.
12. Dissolve the ssRNA in 20 µL DEPC-treated PBS (*see* **Notes 6** and **7**).
13. Mix equal ng amounts of antisense and sense ssRNAs (*see* **Note 8**).
14. Heat the mixture for five minutes at 95°C, and allow for it to cool down slowly to room temperature by switching off the heating block (*see* **Note 6**).
15. Confirm the proper annealing by gel electrophoresis (*see* **Note 6**).
16. Store the dsRNA at -80°C until further use.

5.4.2. Windowing the Eggs

For access to the embryo the eggs are windowed on the 3rd day of incubation (**Figure 5.1A** and **5.2**).

1. Incubate the fertilized eggs in a preincubator at 39°C (*see* **Notes 9** and **10**).
2. After three days of incubation place the egg on the side for at least 30 min before opening to allow the embryo to reposition on top of the yolk.
3. Mark the position of the embryo on the egg shell with a pencil using an Egg-Lume Candler held against the blunt end of the egg.

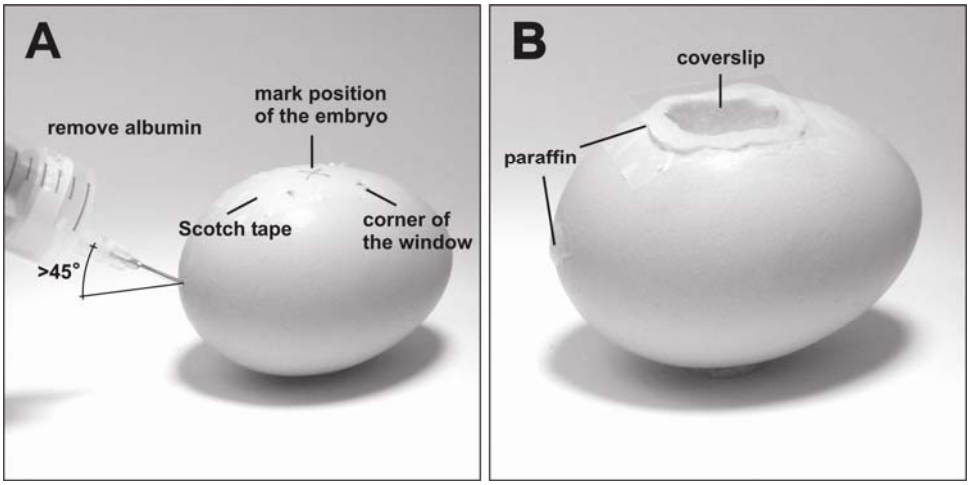

Figure 5.2 Windowing the egg. Before egg windowing, the position of the embryo is marked with a pencil on the shell (A). Subsequently, small holes are drilled at the blunt end of the egg and at the corners outlining the window. Albumin (3 mL) is removed through the hole at the blunt end (*see* **Note 11**). The syringe is kept in an angle of 45° to avoid damage to the embryo and the yolk (A). A piece of Scotch tape prevents pieces of the shell from falling inside the egg while cutting the window with small scissors. For further incubation, the window is sealed with melted paraffin and a coverslip (B).

4. Wipe the eggshell with 70% ethanol to avoid contamination.
5. Make small holes at the blunt end of the egg and at the corners outlining the planned window using a scalpel (**Figure 5.2A**).
6. Carefully remove 3 ml of albumin through the hole at the blunt end of the egg using a syringe (**Figure 5.2A**; *see* **Note 11**).
7. Seal the hole at the blunt end and any possible cracks of the shell by applying melted paraffin.
8. Put a piece of Scotch tape onto the shell to prevent small pieces of the eggshell from falling into the egg (**Figure 5.2A**).
9. Cut the outlined window into the egg shell (*see* **Note 12**).
10. Seal the egg by applying melted paraffin to the edges of the window using a brush and a coverslip (**Figure 5.2B**; *see* **Note 13**).
11. Put the egg back to the incubator. Make sure that the position of the egg is the same as before windowing to keep the embryo accessible through the window.

5.4.3. In ovo Injection and Electroporation

1. Autoclave the tools and wipe the working space with 70% ethanol.
2. Re-open the sealed egg by pressing the soldering iron briefly onto the coverslip and removing it carefully.

3. Stage the embryo according to Hamburger and Hamilton (***21***). Embryos should be between stages 17 and 19.
4. Remove the extraembryonic membranes covering the embryo with forceps and scissors in order to have direct access to the embryo (**Figure 5.3A**).
5. Break off the needle tip to obtain a tip diameter of approximately 5 µm.
6. Sterile PBS containing the dsRNA derived from AXONIN-1 (200-400 ng/µL) and an EGFP reporter plasmid (20 ng/µL) with 0.04 % (vol/vol) Trypan Blue are injected into the central canal of the spinal cord at the level of the hind limbs using a glass capillary connected to a piece of tubing (**Figure 5.1C** and **5.3B**). The injection is controlled by mouth and the maximal injection volume is achieved when the blue dye reaches the brain vesicle (arrowhead in **Figure 5.3B**).
7. Add a few drops of PBS before electroporation to lower electric resistance and to prevent overheating of the embryo.
8. The electrodes are placed in a parallel manner along the anterior-posterior axis of the spinal cord (**Figure 5.3C**).
9. Electroporate the embryo by applying five pulses of 26 V and 50 ms duration each (*see* **Note 14**).
10. After the electroporation, add a few drops of PBS to cool the embryo.
11. Rinse the electrodes with plenty of distilled water to remove denaturated proteins from the surface (*see* **Note 15**).
12. Reseal the egg with a glass coverslip and a soldering iron (*see* **Note 13**).
13. For further incubation the egg is returned to 39°C until stage 25 is reached, i.e., approximately two additional days of incubation ***(21)***.

5.4.4. Analysis of the Phenotype and Electroporation Efficiency

For beginners we recommend to assess the efficiency and reproducibility of the *in ovo* electroporation by analyzing EGFP expression directly *in ovo* under the stereomicroscope (arrowheads in **Figure 5.3D**). Thus, embryos for further analyses can be pre-selected (*see* **Note 16**).
The efficiency as well as the specificity of gene silencing by *in ovo* RNAi can be demonstrated by a variety of approaches. Immunohistochemistry on cryostat sections (***4,13,22***) and Western Blot analysis (***23,24***) are common ways to show downregulation of the targeted protein. If antibodies against the targeted protein are not available, a decrease of the mRNA can be assessed by *in situ* hybridization using either whole-mount embryos (***16,25***) or cryostat sections (***13***). Alternatively, semi-quantitative RT-PCR can be used to detect a decrease of the mRNA (***26***).
Loss-of-function phenotypes can be analyzed in a variety of ways. To study cell differentiation or cell migration, immunohistochemistry for known markers may be a good start (***15***). Changes in morphology and cell positions, expression patterns, etc. can easily be detected. To visualize axonal trajectories staining and/or dye tracing in slices or whole-mount preparations are used to account for their three-dimensionality. For an initial assessment and to localize a specific phenotype within the peripheral nervous system, we recommend a Neurofilament staining of whole-mount preparations ***(24).***

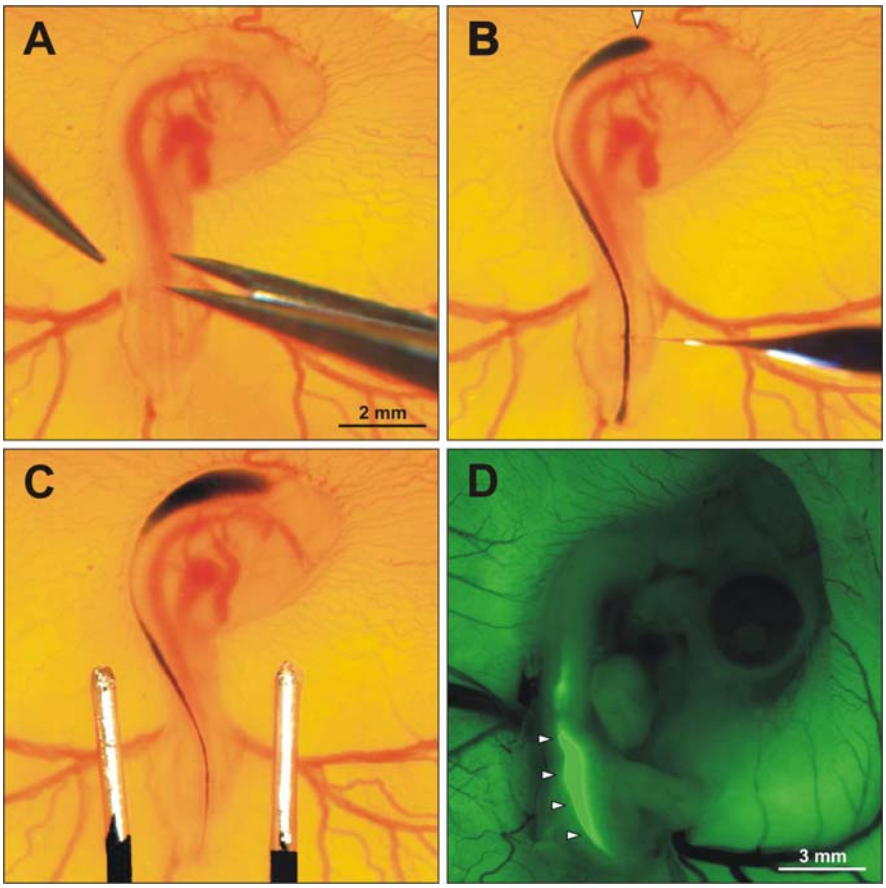

Figure 5.3 Injection and electroporation of the embryo. The extraembryonic membranes covering the embryo are carefully removed before injection using forceps and spring scissors (A). The injection mixture containing dsRNA derived from the gene of interest, an EGFP plasmid for transfection control, and Trypan Blue is injected into the central canal with a glass capillary. The maximal injection volume is achieved when the Trypan Blue has reached the brain vesicle (arrowhead in B). After retraction of the injection needle the electrodes are placed in a parallel manner along the embryonic axis (C). For stage 18 embryos 5 pulses of 26 Volts with 50 ms duration are applied for efficient transfection (*see* **Note 14**). The successful transfection can be verified by the expression of EGFP (indicated by arrowheads) under a stereomicroscope equipped with fluorescence optics 2 days after electroporation (D).

Alternatively or subsequently, mechanisms involved in wiring the nervous system can be analyzed in vibratome slices by dye tracing or immunohistochemistry (***4,14***). For example, we studied molecular mechanisms underlying the pathfinding behavior of commissural axons in the spinal cord using dye tracing in open-book preparations (***4,13,27***).

5.5 Notes

1. Different recipes exist for PBS, the addition of KCl turned out to be crucial for optimal survival rates.
2. The incubation time and developmental progress of the embryo is dependent on the temperature and humidity. Any incubator set at a temperature of 38-39°C can be used, as long as high humidity (at least 45%) and a good air circulation can be achieved. The use of two incubators, one for preincubation and one for treated embryos, is recommended to minimize contaminations and reduce detrimental effects on the treated embryos due to frequent opening and closing of the incubator.
3. Alternatively, any other electroporator that generates square wave pulses can be used (for a comparison of the different electroporators that are commercially available, see ***8***). For different target tissues different electrodes have to be chosen to get best results: For *in ovo* electroporations of the developing spinal cord, we use wire electrodes (***4,13***). Commercially available electrodes can be found at http://www.btxonline.com/products/electrodes/inovo. Alternatively, for a widespread transfection platelet electrodes can be used (***28***). For a small transfection area a needle electrode can be placed directly into the tissue (***17,29,30***).
4. In addition to long dsRNA, short interfering RNAs (siRNA) and short hairpin RNAs (shRNA) have been applied successfully for RNAi in chicken embryos (***14-17,22,31***). In contrast to siRNAs selected by various available algorithms, long dsRNA was always effectively silencing target genes in our hands. Long dsRNA is processed by Dicer to give rise to a large number of siRNAs and therefore will always produce many effective ones making lengthy (and expensive) selection processes unnecessary. Furthermore, long dsRNA can be easily produced by *in vitro* transcription from a cDNA fragment or EST without further cloning steps or expensive synthesis of siRNAs. Chicken ESTs can be obtained from Geneservices Ltd. at http://www.chick.umist.ac.uk. To exclude any off-target effects (silencing of non-target genes) we recommend using two different non-overlapping dsRNA fragments, as it is highly unlikely that they would both have the same off-target effects. In contrast to mammalian cell lines and non-embryonic tissue long dsRNA can be applied to embryonic tissue without induction of unspecific effects (***4,18,19***). No general inhibition of protein synthesis or induction of apoptosis has been reported in mouse oocytes, embryo-derived cell lines, and in chicken embryos (***4, 15, 32, 33***).
5. For *in vitro* transcription RNase-free tips, tubes, and DEPC-treated ddH_2O have to be used. Before starting, clean the workspace with RNaseZAP.
6. Collect 1 µl samples after each step and keep them to control the quality of ssRNA and dsRNA. For this purpose, load the ssRNAs collected after each step and the dsRNA on a 1% agarose gel. Lanes 1 and 2 (one sense and one antisense) contain the linearized plasmids and the ssRNA synthesized by *in vitro* transcription. Lanes 3/4 and 5/6 are sense and antisense ssRNA after DNaseI treatment and purification, respectively. In lane 7 the resulting dsRNA after annealing is loaded. When lanes 5 and 6 are compared to lane 7, the band shift due to the difference in migration between ssRNA and dsRNA should be detected. Furthermore, both ssRNA and dsRNA should give distinct bands. If a smear indicating degradation of the RNA is obtained, the dsRNA should not be used for *in ovo* RNAi.

7. Make sure that the pH and salt concentration of the buffer used to dissolve the ssRNA is in the physiological range and does not have any effect on the development and survival rate of the embryo. Do not use any buffers containing Tris or glycerol.
8. The concentration of the dsRNA for *in vivo* injections should be in the range of 200-400 ng/µL.
9. Eggs should be stored at 15°C for a maximum of one week before incubation. When the eggs are stored for longer periods normal development of the embryo is unlikely.
10. To reach 45% humidity it is usually sufficient to place a tray of distilled water containing 0.1 g/L copper sulfate at the bottom of the incubator. Copper sulfate decreases the risk of contamination.
11. The holes at the corners are required to allow entry of air and detachment of the embryo from the shell during removal of albumin. Insert the syringe at a steep angle (about 45°; **Figure 5.2A**) to avoid damage to the embryo and the yolk which is not compatible with survival.
12. Keep the scissor as horizontally as possible to prevent any damage of the embryo.
13. Make sure that the window is properly closed to prevent dehydration during further incubation. Dehydration will cause the death of the embryo. If the paraffin is cooled down too quickly heat the coverslip briefly with the soldering iron while pressing it down so that the coverslip seals properly along all the edges. Alternatively, the window can be sealed with Scotch tape. Although sealing with coverslips instead of tape is more time-consuming, it facilitates reopening the window and the development of the embryo can directly be observed through the coverslip. The window can easily be reopened by brief heating of the coverslip using a soldering iron.
14. The electroporation settings should be chosen according to the embryonic stage (see also ***12***): For stage 18 embryos 5 pulses of 26 V and 50 ms are applied. The voltage should be adjusted to the embryonic stage and the tissue that is electroporated:

Day of incubation	Embryonic stage	Target tissue	Settings
2	12-14*	Spinal cord and neural crest derivates (for example dorsal root ganglia)	18V
3	18-20**	Spinal cord and floor plate	26V

* Red ink is applied to visualize the embryo. Blue ink should not be used because it interferes with detection of the Trypan Blue that is used to control injection volume and injection site.

** Electroporation at stage 20 or later with the settings mentioned here will prevent transfection of lateral motoneurons.

During electroporation, contact between the electrodes and the major blood vessels as well as with the embryo have to be avoided to prevent severe damage or death of the embryo. Keep the electrodes away from the heart.
15. Remaining proteins on the electrodes interfere with efficient electroporation.

16. The transfection efficiency depends on the time point of the injection, the concentration of the injected nucleic acids, and the electroporation settings. *In ovo* electroporation with the given settings at embryonic stage 18 resulted in a transfection efficiency of approximately 60% of cells within the electroporated area (***4***).

5.6 Troubleshooting list:

Methods	**Problem that might occur**	**Troubleshooting:** *see* **Note**
Synthesis of long dsRNA	Degradation or bad quality of dsRNA	5, 6
Windowing the eggs	Low survival rate	9, 11, 12, 13
	Contamination	2, 10
	Delay in development	2, 9, 13
In ovo injection and electroporation	Low survival rate	1, 3, 7, 8, 13, 14
	Contamination	2, 10
	Inefficient transfection	14, 15, 16

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7. Appendix

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B Curriculum Vitae

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Education

2003- 2007	PhD in the Laboratory of Developmental Neuroscience, Institute of Zoology, University of Zurich Topic: Ex ovo RNAi for Functional Gene Analysis during Neural Development
2000- 2002	Diploma Thesis in Physiol.- Biochemical. Ecology, Institute of Botany, University of Basel Topic: Chilling- induced 1-aminoclycopropane-1-carboxilic Acid Synthase Gene Expression in the Tomato (*Lycoperisicum esculentum*)
1998- 2000	Graduate Studies in Integrative Biology, University of Basel Special fields: Physiol.- Biochemical. Ecology, Plant Physiology, Biology of vertebrates, Neurobiology, Organic Chemistry
1996- 1998	Undergraduate Studies in Integrative Biology and Molecular Biology, University of Basel/ Biocenter Basel
1991- 1995	College (Gymnasium), Fribourg, Typus C

Positions

2002	Scientific Assistant at the Botanical Institute of University of Basel, projects on gene expression patterns in *L. esculentum*
2001	Science et Cité at the Mustermesse Basel
2001	Teaching Assistant in Cell Biology at the Biocenter Basel
2001	Teaching Assistant for Ecological Biochemistry at the Botanical Institute of University of Basel

Awards

2005	Swiss Society of Neuroscience Travel Fellowship for participation at the 35th Annual Meeting of Society for Neuroscience, Washington DC, November 2005

Meetings

2004	NCCR meeting in Konstanz
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2005	SSN meeting in Zürich
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	ZNZ Symposium in Zürich
	SFN meeting in Washington DC
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	SSN meeting in Basel
	FENS meeting in Vienna
	ZNZ Symposium in Zurich
	Meeting on Axon Guidance and Neural Plasticity in Cold Spring Harbor
2007	SSN meeting in Bern
	International chick meeting in Barcelona
	ZNZ Symposium in Zurich

List of publications

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